RNA-DEPENDENT RIBONUCLEOSIDE MONOPHOSPHATE INCORPORATION IN A EUKARYOTE

by

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ABSTRACT

The RNA-dependent incorporation of ribonucleoside monophosphates into a polyribonucleotide product was studied in rat liver, with particular emphasis placed on the incorporation of UMP. The RNA-dependent UMP-incorporating enzyme activity prepared through phosphocellulose chromatography of a crude enzyme fraction from rat liver exhibited the following characteristics *in vitro*: (a) a pH optimum at pH 7.8, (b) no requirement for a monovalent cation, and (c) an absolute requirement for a divalent cation. With $MnCl_2$ as the sole source of divalent cations, the incorporation of UMP was favoured at a concentration of 3.9 mM $MnCl_2$. The enzyme activity appeared *in vitro* to have no RNA specificity. The amount of UMP incorporated appeared to depend more on the number of moles of RNA added to the assay mixture than on the species of RNA added. The observation that various naturally-occurring RNAs, as well as poly(U), stimulated UMP incorporation and the observation that 28S rRNA added to the assay mixture became radioactively labelled suggested that the reaction was primer- rather than template-dependent. The product was susceptible to the action of RNase A, suggesting that UMP was incorporated into polyribonucleotide material. The observation that the product was not susceptible to the action of either DNase I or pronase agreed with this suggestion. Under conditions which were optimal for UMP incorporation, the product appeared to consist of an oligo(U) chain attached to the primer RNA. This chain was on the average 8 UMP residues long.

When the crude enzyme fraction was subjected to phosphocellulose chromatography, the flow-through ("void volume") fraction appeared to contain some factor, distinct from RNA, which stimulated the RNA-dependent UMP-incorporating activity present in the material eluting from the column with 0.6 M KCl-buffer ("0.6 M" fraction). When the "0.6 M" fraction was further fractionated on DEAE-Sephadex A-25, four peaks of RNA-dependent activity eluted at different ammonium sulfate concentrations. At least two of these peaks coincided with DNA-dependent RNA polymerase activities.

The results are discussed in the context of present knowledge of the RNA-dependent incorporation of ribonucleoside monophosphates.

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LIST OF ABBREVIATIONS

RNA	:	ribonucleic acid
A	:	adenine
C	:	cytidine
G	:	guanine
U	:	uracil
NMP	:	ribonucleoside monophosphate
AMP	:	adenosine monophosphate
CMP	:	cytidine monophosphate
GMP	:	guanosine monophosphate
UMP	:	uridine monophosphate
UDP	:	uridine diphosphate
NTP	:	ribonucleoside triphosphate
UTP	:	uridine triphosphate
poly(A)	:	polyadenylic acid
poly(C)	:	polycytidylic acid
poly(G)	:	polyguanylic acid
poly(U)	:	polyuridylic acid
poly(I)	:	polyinosinic acid
poly(dT)	:	polythymidylic acid
N	:	nucleoside
Np	:	nucleotide
HnRNA (or nRNA)	:	heterogeneous nuclear RNA
HMW nRNA	:	high-molecular-weight nuclear RNA (that fraction of the nuclear RNA which is insoluble at 0°C in aqueous 2.5 M NaCl)

LMW nRNA	:	low-molecular-weight nuclear RNA (that fraction of the nuclear RNA which is soluble at 0°C in aqueous 2.5 M NaCl)
rRNA	:	ribosomal RNA
mRNA	:	messenger RNA
tRNA	:	transfer RNA
cRNA	:	complementary RNA
tcRNA	:	translational control RNA
RNP	:	ribonucleoprotein
HnRNP	:	heterogeneous nuclear ribonucleoprotein
mRNP	:	messenger ribonucleoprotein
DNA	:	deoxyribonucleic acid
RNase	:	ribonuclease
DNase	:	deoxyribonuclease
ATA	:	aurintricarboxylic acid
EDTA	:	ethylenediamine tetraacetic acid
Pi	:	orthophosphate
PPi	:	pyrophosphate
SDS	:	sodium dodecyl sulfate
TCA	:	trichloroacetic acid
Tris	:	tris (hydroxymethyl) aminomethane
DEAE-Sephadex	:	diethylaminoethyl-Sephadex
PAG	:	polyacrylamide gel
OD_{260} unit:		that amount of RNA which gives an optical density of 1 at 260 mμ when it is dissolved in 1 ml of solution, and optical density is measured in a cell of 1 cm light path

OD_{280} unit: that amount of protein which gives an optical density of 1 at 280 mμ when it is dissolved in 1 ml of solution, and optical density is measured in a cell of 1 cm light path

$m\Omega^{-1}$: millimho (10^{-3} mho)

S : Svedberg unit (1 S = 1 x 10^{-13} second)

μCi : microcurie (10^{-6} Ci). One curie of radioactive material is that quantity undergoing the same number of disintegrations per second as 1.0 g of radium (3.7×10^{10} s^{-1})

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CHAPTER 1

INTRODUCTION

1.1 The RNA-dependent incorporation of ribonucleoside monophosphates into RNA: an historical perspective.

Among the most significant landmarks in the research on RNA-dependent RNA synthesis is the discovery by Gierer and Schramm (1956) and Fraenkel-Conrat (1956) that ribonucleic acid, as well as deoxy-ribonucleic acid, could serve as the repository of genetic information. They showed that the RNA of the tobacco mosaic virus (TMV) was necessary and sufficient to produce a successful infection in tobacco leaves. Consequently, the viral RNA was replicated in the infected host cells. The study of the molecular mechanisms of RNA virus replication has advanced considerably since then, and a short discussion of it is presented in Section 1.2.

The now well-known enzymatic addition of the -CCA triplet of bases to the 3'-terminal of "soluble" or "transfer" RNA molecules (Zamecnik et al., 1957; Hecht et al., 1958; Herbert, 1958; Harbers and Heidelberger, 1959), mediated by the tRNA-nucleotidyl transferases, is another RNA-dependent incorporation of ribonucleoside monophosphates into an RNA molecule. Although the addition consists of only three residues, the reaction is crucial to cellular metabolism since it renders the tRNAs useable as substrates by the aminocyl-tRNA synthetases (Priess et al., 1961).

Several reports appeared in the 1960s concerning the

incorporation of UMP into RNA through a reaction which was apparently DNA-independent. Among the systems studied were Ehrlich ascites carcinoma cells (Burdon and Smellie, 1961), Landschutz ascites tumour cells (Wykes and Smellie, 1966) and rat liver microsomes (Wilkie and Smellie, 1968a,b). These systems and their products were at the time not very well characterized, but the reports are notable in that they were among the earliest to suggest the possibility of RNA-templated RNA synthesis in "uninfected" eukaryotic cells.

The synthesis of a poly(A) sequence at the 3'-terminal of nuclear and messenger RNAs is a more recent discovery. The potential physiological significance of this reaction is great and it will be examined in more detail in Section 1.4, along with other RNA-primed homopolymer syntheses.

A very recent finding is the RNA-templated homopolymer synthesis catalyzed by DNA-dependent RNA polymerases (nucleosidase-triphosphate: RNA nucleotidyltransferases, E.C. No. 2.7.7.6).

A survey of the relevant literature suggests that the RNA-dependent incorporation of ribonucleoside monophosphates can take place in at least three ways:

(a) a template-dependent incorporation of all four major ribonucleoside monophosphates into a heteropolymeric RNA product having a nucleotide sequence complementary to that of the template;

(b) a template-dependent homopolymer synthesis; and

(c) a primer-dependent terminal addition of a single or several ribonucleoside monophosphates, either as single additions or as

chains of various lengths.

1.2 RNA template-dependent RNA synthesis in virus-infected cells.

The concept of an RNA template-dependent RNA synthesis is firmly established in the case of several RNA viruses, as described below.

1.2.1 Bacteriophages

Among bacteriophages, the system most extensively studied is that derived from Qβ (see below). The single-stranded RNA which constitutes the sole genetic material of Qβ virions serves both as a template for nucleic acid synthesis and as a messenger for virus-specific protein synthesis. The genomic RNA, since it is equivalent to messenger RNA, is conventionally designated as the "plus" strand. The replication of Qβ RNA would appear to proceed along the following lines: the phage RNA is used as a template by the phage-coded polymerase in synthesizing the complementary "minus" strand. At this stage, the polymerase requires membrane-associated host factors. The newly synthesized "minus" strand is then released from the membrane site and used as a template in the simultaneous synthesis of several progeny genomes. At this stage, the viral polymerase no longer requires the presence of host factors (for review of reproduction of RNA bacteriophages, see Eoyang and August, 1974). The minimal requirements for template recognition by Qβ replicase were found by Küppers and Sumper (1975) to be a C-C-C sequence at the 3'-terminus and a second C-C-C sequence in a defined steric position. They found that the corresponding chemical modifications would convert non-template RNAs

to template RNAs. In fact, such great progress has been made in understanding the action of Qβ replicase that the enzyme has recently been brought to synthesize *in vitro* complementary strands of chemically unmodified heterologous RNAs such as 5S, 18S and 28S rRNAs, 9S histone mRNA and rabbit globin mRNA (Palmenberg and Kaesberg, 1974; Obinata *et al.*, 1975; Feix and Hake, 1975; Feix, 1976).

1.2.2 Plant viruses

Among single-stranded RNA viruses which infect plants, some display fascinating peculiarities. The complete genome of an RNA virus should code for at least an RNA replicase, or a part thereof, plus one type of capsid protein; in addition, the RNA should contain a recognition sequence for the replicase.

In complete-genome RNA viruses, infection is followed by translation and replication of the nucleic acid. The replication of tobacco mosaic virus, for example, appears to involve a replicative form (RF) and a replicative intermediate (RI). The RF, a double-stranded molecule, consists of an unbroken strand of viral RNA, the "plus" strand, and its complementary RNA, the "minus" strand. The RI is composed of a double-stranded RNA molecule of the same length as the RF; in addition, this "core" is believed to have single-stranded tails which are presumably mostly "plus" strands in the process of being synthesized. The RI appears to be the immediate precursor of progeny viral RNA.

Certain single-stranded RNA viruses, the split-genome viruses, have their complete genome distributed among several identical protein

shells (eg. brome mosaic virus number 4). In these, more than one of the RNA components is required for replication.

An even more "unorthodox" RNA virus is the tobacco necrosis satellite virus (TNSV). It is thought to lack a replicase-coding gene and to depend upon a concurrent infection with tobacco necrosis virus (TNV) for its replication.

RNA molecules which lack a gene for a replicase and also a gene for a capsid protein have been reported. These are the viroids responsible for potato spindle tuber and certain other plant diseases. The viroid RNA apparently exists as a free nucleic acid with considerable secondary structure. It contains only about 250 nucleotide residues, some of which constitute a recognition site for a replicase. The enzyme responsible for the replication of viroid RNA would necessarily have to be present in "uninfected" host cells. It has been suggested (Siegel and Hariharasubramanian, 1974) that the RNA-dependent RNA polymerase isolated by Astier-Manifacier and Cornuet (1971) from apparently healthy Chinese cabbage leaves is a possible candidate for this activity. Whether this is in fact the case still remains to be elucidated (for review of reproduction of small plant RNA viruses, see Siegel and Hariharasubramanian, 1974).

1.2.3 Animal viruses

As in the case of small plant RNA viruses, the animal picornaviruses (as per International Enterovirus Study Group, 1963) replicate via double-stranded intermediates. The replicative form (RF), consisting of a "plus" strand annealed to its complementary "minus"

strand, is again encountered. Here also, the replicative intermediate (RI), consisting of a double-stranded core with 4-5 single-stranded tails, has been confirmed as a functional intermediate in the synthesis of infectious "plus" strand viral RNA (for review of the reproduction of picornaviruses, see Levintow, 1974).

1.3 RNA template-dependent RNA synthesis in apparently uninfected prokaryotes and eukaryotes.

1.3.1 RNA template-dependent heteropolymer synthesis

Replicase-like enzyme activities presumably of non-viral origin have been reported in several organisms and tissues. The major reports are listed in Table 1.1.

Of the three RNA-dependent RNA polymerase activities listed in Table 1.1 which are reported in bacteria, the first two (Fox et al., 1964; Niyogi and Stevens, 1965a) are in fact known to be due to the corresponding DNA-dependent RNA polymerase. The third (Louis and Fitt, 1972) would appear to be a novel enzyme.

The activity which was purified by Astier-Manifacier and Cornuet (1971) from the chloroplastic fraction of Chinese cabbage leaves was dependent upon the presence of an RNA template. When the proferred RNA template was turnip yellow mosaic virus (TYMV) RNA, the product was 85% resistant to ribonuclease A. Since the enzyme was found in apparently healthy plants, it seemed to be a non-viral replicase. As mentioned earlier, however, the possibility does exist that its "preferred" template is a viroid RNA.

Table 1.1 Replicase-like RNA-dependent RNA polymerase activities presumably of non-viral origin

ORGANISM OR TISSUE	FRACTION	REFERENCE
Micrococcus luteus	------	Fox *et al.* (1964)
Escherichia coli	------	Niyogi and Stevens (1965a)
Halobacterium cutirubrum	------	Louis and Fitt (1972)
Chinese cabbage leaves	chloroplasts	Astier-Manifacier and Cornuet (1971)
Rabbit reticulocytes	microsomes	Downey *et al.* (1973)
Spleens of mice immunized with *Salmonella tennessee* flagella	------	Saito and Mitsuhashi (1973)
Guinea pig spleen	------	Cramer (1974)
Rat brain	------	Mikoshiba *et al.* (1974)
Rat liver	microsomes	Wilkie and Smellie (1968b)
Rat liver	crude protein fraction obtained from whole cells	Naora (1975)
Cultured human peripheral lymphocytes	------	Neuhoff *et al.* (1970)

Downey et al. (1973) reported the purification of an enzyme which could offer a means of post-transcriptional regulation of globin gene expression in rabbit reticulocytes. The existence of a globin mRNA replicase is certainly appealing, especially in view of the fact that their work utilized enucleate cells, but some doubt has recently been cast upon the validity of their interpretation. Longacre and Rutter (1977) have suggested that Downey et al. may in fact be dealing with an RNA-dependent poly(U)- and poly(C)-synthesizing activity rather than a true replicase.

RNA replicating enzymes have been reported in immunocompetent cells (Neuhoff et al., 1970; Saito and Mitsuhashi, 1973; Cramer, 1974). These replicases are thought to utilize "informational RNA" ("iRNA") as template. The division of opinion among immunologists as to the role and even the existence of such an RNA species makes the assessment of these reports doubly difficult and hazardous. On the one hand, they may be construed as evidence that there is such a functional polyribonucleotide as "informational RNA". On the other hand, unrecognized elements in the rather crude extracts of immunocompetent tissues utilized may be responsible for a very misleading artefact. These reports must accordingly be accepted with reservations.

Mikoshiba et al. (1974) demonstrated the RNA-dependent synthesis of both heteropolymers and homopolymers in rat brain. Unfortunately, their enzyme preparation was crude and their reasons for suggesting the existence of an RNA replicase are not compelling. Since the incorporation of one labelled ribonucleoside monophosphate was reduced in the absence of the other NTPs, as compared with the

incorporation when all were present, they inferred, but did not demonstrate, a template-dependent activity. The report of Wilkie and Smellie (1968b) on a rat liver microsome RNA-dependent RNA polymerase is couched in milder terms than some others and suggests that:

> "Although the reactions seem to lead predominantly to the synthesis of homopolymers the possibility of some formation of some heteropolymer is not excluded."

Naora (1975) partially characterized an enzyme fraction from rat liver which could promote the incorporation of ^{3}H-UMP into TCA-precipitable material. The reaction was stimulated by the addition of nuclear RNA. He showed that a small degree of hybridization could take place between the RNA products and the added nuclear RNA. This suggested a template function for the added RNA.

It can be readily appreciated that there are very few unambiguous reports of _bona fide_ RNA replicases of non-viral origin.

1.3.2 RNA template-dependent homopolymer synthesis

Another way in which RNA-dependent ribonucleoside monophosphate incorporation can take place is by the template-dependent synthesis of ribohomopolymers. Both prokaryotic (Krakow and Ochoa, 1963; Niyogi and Stevens, 1965a; Denisova _et al._, 1975) and eukaryotic (Ballard and Williams-Ashman, 1966) DNA-dependent RNA polymerases (nucleosidasetriphosphate: RNA nucleotidyltransferases, E.C. No. 2.7.7.6) would appear to be capable of synthesizing _in vitro_ a ribohomopolymer complementary to a proferred ribohomopolymer template. There have also been suggestions (Brishammar and Juntti, 1975; Mizuochi and Fukasawa, 1976) that other enzymes exist which are capable of catalyzing this reaction.

The physiological significance of RNA-templated homopolymer synthesis is presently unknown. Recent reports, however, suggest that poly(A)·oligo(U) duplexes may be of some physiological importance, for example in maintaining the tertiary structure of certain RNA molecules. Following their observation that "poly(A)"-protein complexes isolated from HeLa cell HnRNP (heterogeneous nuclear ribonucleoprotein) or mRNP (messenger ribonucleoprotein) particles contained a substantial fraction of UMP residues, Kish and Pederson (1975, 1976) identified oligo(U) sequences 15-50 nucleotides in length in HeLa cell HnRNA. These sequences were base-paired with poly(A), and the poly(A)·oligo(U) duplexes were an aspect of native HnRNP, as opposed to resulting from intra- or intermolecular reassociation during isolation. Kish and Pederson (1977) also suggested that similar duplexes may be present in polysomal mRNA. Whether these poly(A)·oligo(U) duplexes are the result of RNA template-dependent homopolymer synthesis still remains to be elucidated.

1.4 RNA primer-dependent incorporation of ribonucleoside monophosphates.

RNA primer-dependent incorporation of ribonucleoside monophosphates has been reported extensively in both prokaryotes and eukaryotes.

1.4.1 Prokaryotes

The major reports of prokaryotic RNA primer-dependent incorporation of ribonucleoside monophosphates are listed in Table 1.2. Except for one instance where the synthesis of mixed polymers of AMP and GMP is reported (Milanino and Chargaff, 1973), all reactions lead to the formation of homopolymers.

Table 1.2 Prokaryotic RNA primer-dependent incorporation of ribonucleoside monophosphates

ORGANISM	REACTION	REFERENCE
Escherichia coli	poly(A) synthesis	August et al. (1962) Colvill and Terzi (1968) Gottesman et al. (1962) Hardy and Kurland (1966) Milanino and Chargaff (1973) Schäfer et al. (1972) Ohasa and Tsugita (1976)
	poly(U) synthesis	Schäfer et al. (1972)
	poly(C) synthesis	Schäfer et al. (1972)
	synthesis of mixed polymers of AMP and GMP	Milanino and Chargaff (1973)
Pseudomonas putida	poly(A) synthesis	Payne and Boezi (1970)
Shigella dysenteriae	poly(A) synthesis	Colvill and Terzi (1968)

The significance of an AMP-polymerizing enzyme activity in prokaryotes, which lack the heterogeneous nuclear RNA characteristic of eukaryotes, has long remained obscure. Recently, however, poly(A) sequences have been identified in Escherichia coli messenger RNA (Nakazato et al., 1975), where they may serve the same function as is attributed to the 3'-terminal poly(A) segment of eukaryotic messenger RNAs (see Section 1.4.3). Although the poly(A) polymerization in E. coli was initially thought to be catalyzed by the α constitutive subunit of the bacterial DNA-dependent RNA polymerase (Ohasa and Tsugita,

1972; Ohasa et al., 1972), further purification has revealed that the activity is in fact due to a novel enzyme (Ohasa and Tsugita, 1976). Similarly, the other bacterial poly(A) polymerases listed in Table 1.2 are thought to be enzymes distinct from the corresponding DNA-dependent RNA polymerase. Very little is known at present concerning the physiological role, if any, of polymers and oligomers of UMP, CMP or GMP in prokaryotes.

1.4.2 Eukaryotes

The major reports of eukaryotic RNA primer-dependent incorporation of ribonucleoside monophosphates are listed in Tables 1.3 - 1.6. As can be seen, they deal mainly with homopolymer synthesis. Other reactions, such as the synthesis of the 3'-terminal -CCA on tRNA, are included because of their RNA primer requirement.

1.4.3 Functional significance of homopolymers

Among RNA-primed homopolymer synthetic reactions in eukaryotes, poly(A) polymerization at the 3'-end of HnRNA and mRNA has received the most attention. The presence of poly(A) sequences in some HnRNA molecules is now well documented (Darnell et al., 1971; Edmonds et al., 1971; Greenberg and Perry, 1972; Jelinek et al., 1973). These sequences are located at the 3'-terminal of HnRNA (eg. Molloy et al., 1974; Herman et al., 1976). The nuclear RNA of the cellular slime mould Dictyostelium discoideum, exceptionally, contains an additional poly(A)$_{25}$ segment which is internal to the molecule and separated from the 3'-terminal poly(A)$_{100}$ by about 10 to 40 nucleosides (Lodish et al., 1974). This internal poly(A)$_{25}$ is apparently transcribed from poly(dT)$_{25}$

Table 1.3 Eukaryotic RNA primer-dependent incorporation of adenosine monophosphate

TISSUE	REACTION	CELL FRACTION (where specified)	REFERENCE
1. Rat liver	poly (A) synthesis	nuclei	Niessing and Sekeris (1972, 1973, 1974)
			Rose and Jacob (1974)
			Niessing (1975)
		mitochondria	Jacob and Schindler (1972)
		microsomes	Page et al. (1967)
		--	Klemperer (1963a, 1964, 1965)
	synthesis of 3'-terminal -CCA on tRNA	105000 x g supernatant	Zamecnik et al. (1957)
		--	Hecht et al. (1958)
		--	Herbert (1958)
		--	Harbers and Heidelberger (1959)
2. Rat brain	poly (A) synthesis	nuclei	Dravid et al. (1971)
		non-nuclear particulate fraction	Gurgo et al. (1970)
3. Mouse sarcoma	elongation of poly (A) sequence at 3'-end of nuclear and mRNA	--	Brawerman and Diez (1975)
4. Landschutz ascites	synthesis of short homopolymers of AMP	microsomes	Wykes and Smellie (1966)

Table 1.3 (continued)

TISSUE	REACTION	CELL FRACTION (where specified)	REFERENCE
5. Chinese hamster cells	elongation of poly (A) sequence at 3'-end of nuclear and mRNA	--	Brawerman and Diez (1975)
6. Calf thymus	poly (A) synthesis	nuclei	Edmonds and Abrams (1960, 1962, 1963)
			Winters and Edmonds (1973)
		cytoplasm	Tsiapalis et al. (1973)
7. Avian erythro-cytes	poly (A) synthesis	--	Longacre and Rutter (1977)
		--	Appels and Williams (1970)
8. Sea urchin embryos	poly (A) synthesis	nuclei	Hyatt (1967)
9. Wheat	poly (A) synthesis	chloroplasts	Burkard and Keller (1974)
10. Yeast	poly (A) synthesis	ribosomes	Twu and Bretthauer (1971)

Table 1.4 Eukaryotic RNA primer-dependent incorporation of uridine monophosphate

TISSUE	REACTION	CELL FRACTION (where specified)	REFERENCE
1. Rat liver	poly (U) synthesis	nuclei	Niessing and Sekeris (1974)
			Niessing (1975)
		microsomes	Page et al. (1967)
		--	Klemperer (1963b)
	terminal addition of only 2-3 uridine residues	105000 x g supernatant	Wilkie and Smellie (1968b)
2. Rat brain	poly (U) synthesis	--	Hozumi et al. (1975)
3. Ehrlich ascites	poly (U) synthesis	--	Burdon and Smellie (1961)
4. Landschutz ascites	synthesis of short homopolymers of UMP	microsomes	Wykes and Smellie (1966)
5. Avian erythrocytes	poly (U) synthesis	ribosomes	Boyd and Fitschen (1975)
		--	Appels and Williams (1970)

Table 1.5 Eukaryotic RNA primer-dependent incorporation of cytidine monophosphate

TISSUE	REACTION	CELL FRACTION (where specified)	REFERENCE
1. Rat liver	poly (C) synthesis	nuclei	Niessing and Sekeris (1974)
			Niessing (1975)
		microsomes	Page et al. (1967)
		--	Klemperer (1963b)
	synthesis of 3'-terminal -CCA on tRNA	105000 x g supernatant	Zamecnik et al. (1957)
		--	Hecht et al. (1958)
		--	Herbert (1958)
		--	Harbers and Heidelberger (1959)
2. Rat brain	poly (C) synthesis	nuclei	Mandel et al. (1967)
3. Landschutz ascites	synthesis of short homopolymers of CMP	microsomes	Wykes and Smellie (1966)
4. Calf thymus	poly (C) synthesis	nuclei	Edmonds and Abrams (1960)
			Edmonds (1965)
5. Avian erythrocytes	poly (C) synthesis	ribosomes	Boyd and Fitschen (1975)

Table 1.6 Eukaryotic RNA primer-dependent incorporation of guanosine monophosphate

TISSUE	REACTION	CELL FRACTION (where specified)	REFERENCE
1. Rat liver	poly (G) synthesis	nuclei	Niessing and Sekeris (1974)
			Niessing (1975)
		microsomes	Page et al. (1967)
		--	Klemperer (1963b)
2. Ehrlich ascites	incorporation of GMP into tRNA	--	Itoh et al. (1975)
3. Landschutz ascites	synthesis of short homopolymers of GMP	microsomes	Wykes and Smellie (1966)
4. Wheat	poly (G) synthesis	chloroplasts	Burkard and Keller (1974)
5. Spinach	poly (G) synthesis	chloroplasts	Chakravorty and Biswas (1965)

sequences in Dictyostelium DNA by a DNA-dependent RNA polymerase.

The terminal poly(A) sequences appear not to be transcribed from DNA, but rather to be post-transcriptionally added to HnRNA (Darnell et al., 1973). The addition of a poly(A) segment at the 3'-end of nuclear RNA apparently takes place in two distinct stages. The first is the synthesis of a 150-200 nucleotide-long segment in a reaction which is sensitive in cell culture to cordycepin (3'-deoxy-adenosine). The second consists of the addition of a further 6-8 residues in a reaction which is cordycepin-insensitive (Diez and Brawerman, 1974).

Enzymes capable of poly(A) synthesis have been isolated from rat liver nuclei (Niessing and Sekeris, 1972, 1973, 1974; Rose and Jacob, 1974; Niessing, 1975), rat brain nuclei (Dravid et al., 1971), calf thymus nuclei (Edmonds and Abrams, 1960, 1962, 1963; Winters and Edmonds, 1973) and the nuclei of sea urchin embryos (Hyatt, 1967). The function of the 3'-poly(A) segment of HnRNA has not been ascertained, but it is thought to play a role in the processing of HnRNA to messenger RNA and its transport from nucleus to cytoplasm (for review, see Lewin, 1974).

The presence of a poly(A) sequence at the 3'-end of messenger RNA, except in the case of histone mRNA, is also well documented (eg. Adesnik et al., 1972; Dina et al., 1974; Gorski et al., 1974). This fact has long been considered indirect evidence for the precursor-product relationship between HnRNA and mRNA. There are now indications, however, that these sequences may be newly synthesized in the cytoplasm

(Tsiapalis et al., 1973; Brandhorst and McConkey, 1975; Hadidi and Sethy, 1976). Although this does not necessarily detract from the possibility of an HnRNA-mRNA precursor-product relationship, it is evident that the actual significance of the existence of a 3'-poly(A) sequence on both RNA species will have to be reassessed. The discovery by Brawerman and Diez (1975) that the 3'-poly(A) sequence of both nuclear and messenger RNAs in mammalian cells is subject to an elongation process distinct from de novo synthesis illustrates the possibility that, although mRNA may be derived from HnRNA, the 3'-poly(A) sequence of mRNA may at least in part be distinct from the 3'-poly(A) sequence of HnRNA. Whatever its origin, the 3'-poly(A) segment of mRNA apparently has no direct effect on protein synthesis (Williamson et al., 1974) but may confer some nuclease resistance upon cytoplasmic mRNAs and thus increase their functional lifetime (Huez et al., 1974; Levy et al., 1975).

The physiological role, if any, of the other three ribohomopolymer sequences is still obscure, although U-rich RNAs are attracting an increasing amount of interest. The existence of oligo(U) regions, 20-30 UMP residues in length, within HnRNA molecules is now well accepted (Burdon and Shenkin, 1972; Molloy et al., 1974; Dobrzanska and Buchowicz, 1976). Although these regions are apparently transcribed from DNA (Molloy et al., 1972), other RNA species have been reported which contain U-rich regions of undetermined origin. For example, low-molecular-weight nuclear RNAs are known to stimulate DNA-dependent RNA synthesis, presumably due to specific structures which have a high content of uridylic acid (Kanehisa et al., 1974). Also, small U-rich cytoplasmic RNAs have been imputed with a function in

translational control (Bogdanovsky et al., 1973; Bester et al., 1975; Lee-Huang et al., 1976). Burdon et al. (1977) have recently discovered a metabolically unstable oligo(U)-containing RNA which appears to be a specific subclass of HnRNA. They also found a metabolically unstable cytoplasmic oligo(U)-containing RNA which is similar in size to poly-adenylated messenger RNAs and which associates with polysomes.

Whether oligo(U)-containing RNAs play a vital role in the biochemistry of the cell has not yet been demonstrated. By comparison with the poly(A)-containing RNAs, however, they have been relatively poorly studied. This is probably because of the difficulties involved in obtaining these RNAs. Some are metabolically unstable (Burdon et al., 1977) while othersare of such a small size that, even though their percentage content of UMP residues is high, they will not hybridize extensively with poly(A) which has been immobilized on ion-exchangers.

The in vitro addition of poly(U) and oligo(U) chains onto RNA primers and the purification, to varying degrees, of the enzyme responsible have been reported in various tissues and cells: rat liver (Klemperer, 1963b; Page et al., 1967; Wilkie and Smellie, 1968a,b; Niessing and Sekeris, 1974; Niessing, 1975), rat brain (Hozumi et al., 1975), Ehrlich ascites cells (Burdon and Smellie, 1961), Landschutz ascites cells (Wykes and Smellie, 1966) and avian erythrocytes (Appels and Williams, 1970; Boyd and Fitschen, 1975; Longacre and Rutter, 1977).

The enzymatic capability of eukaryotes for synthesizing RNAs containing U-rich regions has thus been demonstrated and the potential significance of RNA molecules containing U-rich segments has been signaled.

1.5 RNA-dependent ribonucleoside monophosphate incorporation: major objectives of the study.

The present study was undertaken for the purpose of characterizing a eukaryotic enzyme activity promoting the RNA-dependent incorporation of ribonucleoside monophosphates into a TCA-precipitable product. For reasons which will be given in the text, uridine triphosphate was chosen as the labelled NTP for this characterization.

Earlier studies (Wilkie and Smellie, 1968a,b; Naora, 1975) on the RNA-dependent incorporation of ^{3}H-UMP in "uninfected" eukaryotes indicated that although an RNA primer-dependent activity predominated, the possibility existed that an RNA template-dependent activity might also be present. As these *in vitro* studies were conducted with crude extracts, there was an obvious need for an improved preparation method and a more detailed characterization of the enzyme(s) involved.

Possible enzyme-mediated reactions leading to the RNA-dependent incorporation of UMP into TCA-precipitable material were:

(a) template-dependent heteropolymer synthesis. The demonstration of RNA amplification in "uninfected" eukaryotes would of course constitute a breakthrough in the field of post-transcriptional regulation of mammalian gene expression.

(b) template-dependent or primer-dependent homopolymer synthesis. The characterization of either of these reactions and its product might assist in clarifying the origin and/or function of oligo(U) sequences found in or in association with certain cellular RNAs. That such sequences exist has been demonstrated (see Section 1.4.3)

but their origin and their role have not been elucidated. In this respect, the present study might well reveal a hitherto unappreciated modulation of nucleic acid function.

(c) terminal addition onto RNA molecules of single nucleotide residues. The biological significance of such terminal additions is presently unknown. It is possible, however, that this reaction is involved in the post-transcriptional modulation of nucleic acid function.

The specific aims of the present study consist of:

(a) obtaining a partially purified enzyme fraction which can demonstrably promote the RNA-dependent incorporation of UMP into a TCA-precipitable product (Chapter 3).

(b) characterizing the enzyme activity, in terms of pH and ionic requirements, RNA specificity and substrate (NTP) preference (Chapter 4).

(c) characterizing the major reaction product, including a demonstration that this product is a polyribonucleotide, the determination of whether this product is a heteropolymer or a homopolymer, the determination of whether the added RNA acts as a template or as a primer and a determination of the chain length of the newly synthesized segment (Chapter 5).

(d) finally, obtaining some indication of the relationship of the enzyme activity to the cellular DNA-dependent RNA polymerase activities (Chapter 6).

CHAPTER 2

MATERIALS AND METHODS

The methods described in this chapter are relevant to several Results chapters. Methods which are relevant to only one chapter are described in that chapter.

2.1 Animals

In order to reduce the possibility of contamination of the different preparations by viral or bacterial enzymes, specific pathogen-free (SPF) rats were used throughout the experiments. These were male Wistar rats weighing 160-180g. Rabbits were multi-coloured semi-lop and mice were Hall Institute-derived outbred.

2.2 Glassware

All glassware, including chromatographic columns and micro-pipettes (H.E. Pederson, Denmark), was chromic acid-washed before use to destroy RNase activity.

2.3 Chemicals

Unless otherwise specified, chemicals were analytical grade. Sucrose was Wako (Osaka) "saccharose" unless ribonuclease-free sucrose was required, in which case density gradient grade sucrose from Schwartz/Mann (New York) was utilized. Tris (base), ultra pure, was from Schwartz/Mann. All aqueous solutions were prepared in glass-distilled water.

2.4 Dispensing

Dispensing of solutions was carried out using B grade pipettes for volumes of 0.2 ml or greater. Smaller volumes were transferred using glass micropipettes, Oxford Samplers (Oxford Laboratories, California) or a Gilson adjustable Pipetman P20 (Gilson, France), the latter two with disposable Teflon tips.

2.5 Balances

Chemicals and other material were weighed on either a Mettler P-120 (Mettler, Switzerland) or a Mettler H20 balance. Tubes were balanced prior to centrifugation using a Harvard trip balance (Ohaus Scale Corporation, New Jersey).

2.6 Buffers

Buffers were adjusted to the required pH at room temperature using a PHM62 standard pH meter (Radiometer, Copenhagen). Either hydrochloric acid (Ajax Chemicals, Melbourne) or ammonia solution (Ajax Chemicals, Melbourne) were added to reach the desired pH.

2.7 Centrifuges

Low speed centrifugation (2000 x g or less) was carried out in a Sorvall GLC-1 centrifuge (Newtown, Connecticut) with the HL-4 rotor or in the Clements GS200 (H.I. Clements Pty. Ltd., New South Wales) with the U200 Universal 4 place swing-out head.

High speed centrifugation was performed with the following

centrifuges and rotors:

(1) Sorvall RC2-B centrifuge with the GSA, SS-34 and HB-4 rotors.

(2) Sorvall SS-3 centrifuge with the GSA, SS-34 and HB-4 rotors.

(3) Beckman Model L centrifuge (Beckman, California) with the 21 rotor.

(4) Beckman L2-65B ultracentrifuge with the SW 27 rotor.

2.8 Protein determination

The method used to determine the protein concentration in the enzyme preparations was that of Lowry *et al*. (1951). The preparations, or aqueous dilutions thereof, were incubated at 37°C for 2 hours with equal volumes of 2 N sodium hydroxide. Further dilutions were then performed when required with 1 N sodium hydroxide.

After the reaction with Folin and Ciocalteu's reagent (Ajax Chemicals, Melbourne) was completed, the optical density of the samples was read in 2 ml glass cuvettes at 750 mμ with a Gilford (Ohio, U.S.A.) 240 spectrophotometer.

In each determination, standards containing 0, 12.5, 25, 37.5 and 50 μg of bovine serum albumin (The Armour Laboratories, Kankanee, Illinois) received the same additions at the same time as the samples. The protein content of the enzyme preparations could then be determined using this calibration curve.

2.9 Determination of ribonucleic acid content

Samples or aqueous dilutions thereof were placed in 2 ml quartz cuvettes and their optical density was determined at 260 mμ with a Gilford 240 spectrophotometer. Concentrations were calculated according to the formula:

$$1\ OD_{260} = 33.3\ \mu g\ RNA$$

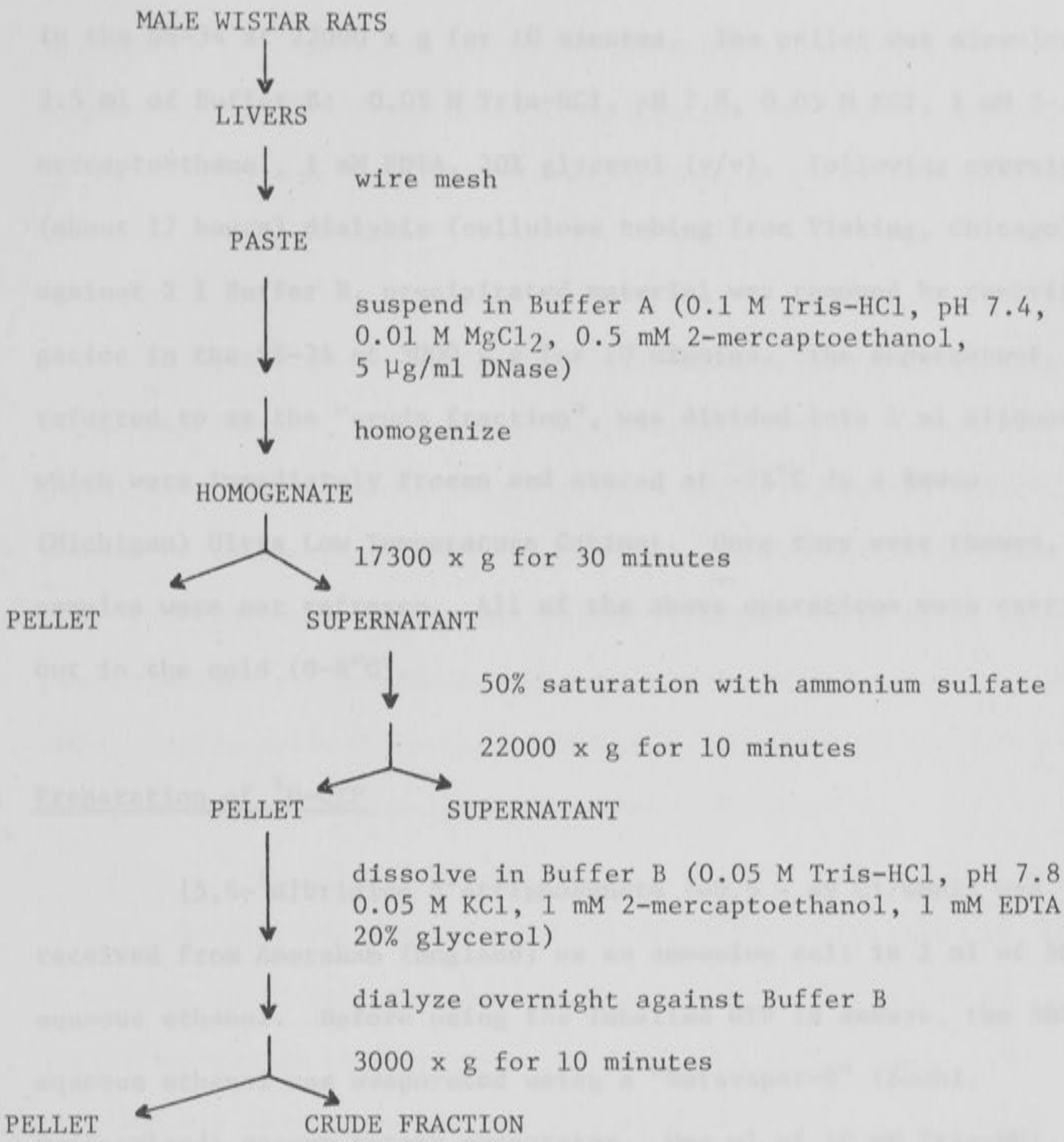
The OD_{260}/OD_{280} ratio was used as an index of RNA purity.

2.10 Preparation of the crude enzyme fraction

The method of preparation was a modification of that utilized by Haruna et al. (1972) in preparing RNA-dependent RNA polymerase from leukemia and ascites tumour cells. It is outlined in Figure 2.1.

Male Wistar rats weighing 160-180 g were sacrificed and the livers removed. These were passed through a wire mesh (354 μ). The paste was suspended in Buffer A: 0.1 M Tris-HCl, pH 7.4, 0.01 M $MgCl_2$, 0.5 mM 2-mercaptoethanol, 5 μg/ml DNase (Sigma, St. Louis). Approximately 40 ml Buffer A were used for 4 whole livers (25 g wet weight). The suspension was homogenized in Thomas tissue grinders (Philadelphia) with 6 strokes at setting 8 on the Caframo (Ontario, Canada) RZR1-64 stirrer using a loose plunger with a Teflon tip. The homogenate was centrifuged at 17300 x g for 30 minutes in the Sorvall RC2-B with the SS-34 rotor cooled to 4°C. An equal volume of saturated ammonium sulfate (Malinckrodt, St. Louis), adjusted immediately beforehand to pH 7.4, was added dropwise to the supernatant. Precipitation was allowed to take place for 2 hours on ice.

Figure 2.1 Preparation of the crude enzyme fraction.


MALE WISTAR RATS
LIVERS
wire mesh
PASTE
suspend in Buffer A (0.1 M Tris-HCl, pH 7.4, 0.01 M MgCl2, 0.5 mM 2-mercaptoethanol, 5 µg/ml DNase)
homogenize
HOMOGENATE
17300 x g for 30 minutes
PELLET
SUPERNATANT
50% saturation with ammonium sulfate
22000 x g for 10 minutes
PELLET
SUPERNATANT
dissolve in Buffer B (0.05 M Tris-HCl, pH 7.8, 0.05 M KCl, 1 mM 2-mercaptoethanol, 1 mM EDTA, 20% glycerol)
dialyze overnight against Buffer B
3000 x g for 10 minutes
PELLET
CRUDE FRACTION

The salt-precipitated material was collected by centrifugation in the SS-34 at 22000 x g for 10 minutes. The pellet was dissolved in 2.5 ml of Buffer B: 0.05 M Tris-HCl, pH 7.8, 0.05 M KCl, 1 mM 2-mercaptoethanol, 1 mM EDTA, 20% glycerol (v/v). Following overnight (about 17 hours) dialysis (cellulose tubing from Visking, Chicago) against 2 1 Buffer B, precipitated material was removed by centrifugation in the SS-34 at 3000 x g for 10 minutes. The supernatant, referred to as the "crude fraction", was divided into 1 ml aliquots which were immediately frozen and stored at -75°C in a Revco (Michigan) Ultra Low Temperature Cabinet. Once they were thawed, samples were not refrozen. All of the above operations were carried out in the cold (0-8°C).

2.11 Preparation of ^{3}H-UTP

[5,6-^{3}H]Uridine 5'-triphosphate (40.5 - 49 Ci/mmol) was received from Amersham (England) as an ammonium salt in 1 ml of 50% aqueous ethanol. Before using the labelled UTP in assays, the 50% aqueous ethanol was evaporated using a "Rotavapor-R" (Büchi, Switzerland) vacuum rotary evaporator. One ml of 10 mM Tris-HCl, pH 7.5, was then added to the vials. The ^{3}H-UTP, thus dissolved in Tris buffer, was stored in a Kelvinator freezer at -20°C.

2.12 Method of enzyme assay

The standard assay mixture contained in 0.125 ml: 83 mM Tris-HCl, pH 7.8, 5 µCi (40.5 - 49 Ci/mmol) ^{3}H-UTP, 0.08 mM UTP, 10 µg/ml actinomycin D, 3.9 mM $MnCl_2$, 10 mM $MgCl_2$, 0.11 mM 2-mercaptoethanol and 6 µg nRNA. Departures from the concentrations listed above, as well as the addition of protein from the enzyme fraction

and the addition of other components (eg. NTPs), are listed in the text or in the legends to the appropriate Tables and Figures. In all cases, the ^{3}H-NTP was added last. Unless otherwise indicated, ^{3}H-UTP (5 μCi) was used throughout the experiments as the labelled NTP.

Unlabelled ribonucleoside triphosphates were obtained from Sigma (St. Louis). Adenosine 5'-triphosphate ($Na_2 \cdot 3.5H_2O$) and guanosine 5'-triphosphate ($Na_3 \cdot 0.5H_2O$) were from equine muscle; uridine 5'-triphosphate ($Na_3 \cdot 2.5H_2O$) and cytidine 5'-triphosphate ($Na_3 \cdot 2.0H_2O$) were from yeast. Stock solutions (0.01 M) of these ribonucleoside triphosphates were adjusted to pH 7.5 and stored at -20°C. Actinomycin D was from Calbiochem (California) and 2-mercapto-ethanol was from Eastman Kodak Co. (New York).

Incubations were carried out in lipped 15 ml tubes at 36°C in a Haake water bath (Gebrüder Haake, Berlin). The period of incubation was 20 or 60 minutes, as indicated in the text. The reaction was stopped by the addition of 0.15 ml of a 1.5% sodium pyrophosphate (Ajax Chemicals, Sydney) - 0.5 M sodium dihydrogen phosphate (B.D.H. Chemicals Ltd., England) mixture. Approximately 50 μg of whole cell RNA from rat liver was added as carrier, followed by sodium dodecyl sulfate (SDS) (Matheson, Coleman and Bell, California) to a final concentration of 1%. A further 3 minute incubation at 36°C in the presence of 1% SDS was found to reduce the "background" radioactivity. The tubes were then placed on ice and cold 50% trichloroacetic acid (TCA) (Ajax Chemicals, Melbourne) was added to a final concentration of 5%.

The "zero-time" control consisted of a mixture identical to the others and incubated at 36°C along with them, but with ^{3}H-UTP withheld until the end of the incubation period. One minute before the end of the incubation period, the sodium pyrophosphate-sodium dihydrogen phosphate mixture was added to the "zero-time" tube. This was immediately followed by 5 µCi ^{3}H-UTP, 50 µg carrier RNA and 1% SDS. The "zero-time" tube was also incubated for 3 minutes at 36°C in the presence of 1% SDS.

Precipitation of the carrier RNA and labelled material was allowed to take place in 5% TCA for 20 minutes on ice. The incubation mixtures were then filtered using Whatman (England) GF/C glass fiber discs on a vacuum filtration apparatus with 18 psi vacuum pressure. The tubes were each rinsed with 35 ml ice-cold 5% TCA in 5 ml aliquots. The filters were then placed in glass vials (Beckman, California) and dried under a heat lamp.

2.13 Measurement of radioactivity

One ml of NCS (Amersham/Searle, Illinois) tissue solubilizer containing 10% water was added to each vial (see Section 2.12). The vials were placed in a 37°C incubator for 2 hours. Ten ml of Malinckrodt (St. Louis) toluene-based scintillation fluid containing 2,5-diphenyloxazole (Searle Nucleonics, Sydney) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (Packard, Illinois) (PPO-POPOP) were then added. The radioactivity in each vial was determined with a Beckman LS-100 liquid scintillation counter. The counting efficiency of the LS-100 was 35%. The radioactivity was expressed in terms of "counts per minute".

Malinckrodt (St. Louis) rather than Univar (Ajax Chemicals, Melbourne) toluene was used in preparing the scintillation fluid, as the latter was found to cause quenching.

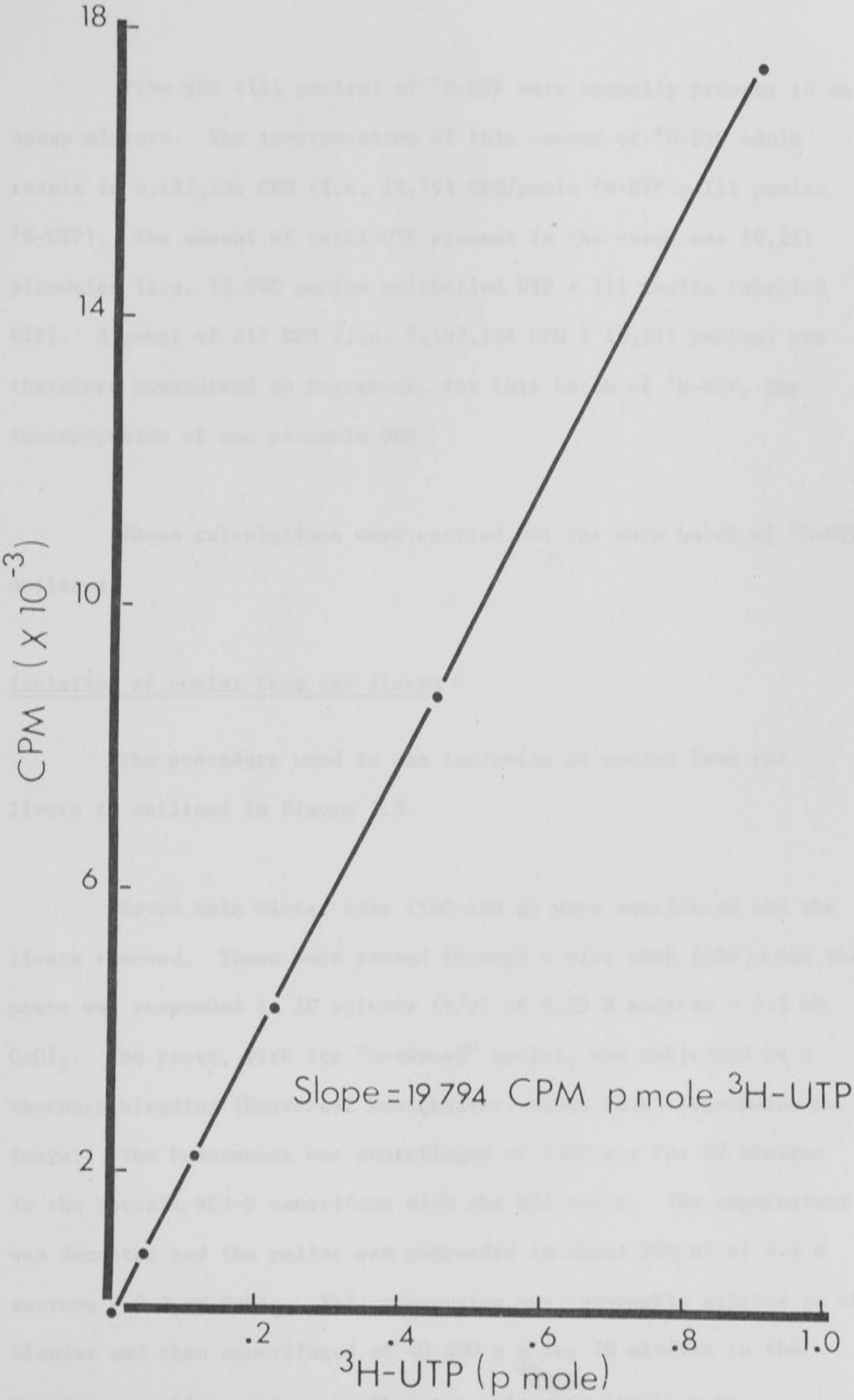
2.14 Determination of picomoles UMP incorporated

A 1:5000 dilution (1 µl in 5 ml) of ^{3}H-UTP in 10 mM Tris-HCl, pH 7.5 (see Section 2.11), was made. Small aliquots of this dilution were placed directly onto GF/C filters in scintillation vials. The filters were dried under a heat lamp and treated with NCS. Radioactivity was determined as described above. Since both the specific activity and the concentration of ^{3}H-UTP in the undiluted stock were known, it was possible to calculate the amount of ^{3}H-UTP present in each vial. Plotting the radioactive counts per minute against the amount of ^{3}H-UTP present in each vial showed a linear relationship. The slope of the line represented "counts per minute per picomole ^{3}H-UTP present" (see Figure 2.2).

Adding only 5 µCi ^{3}H-UTP to each assay tube meant that the ^{3}H-UTP concentration was 0.67-1.00 µM. The labelled UTP was supplemented with a known amount of unlabelled UTP, for a final "total UTP" (labelled + unlabelled) concentration of 0.081 mM.

Since the amount of total UTP present in the assay mixture was known, it was possible to calculate the radioactive counts per minute which would result from the incorporation of one picomole UMP (total UMP, i.e. labelled + unlabelled). These calculations were based on the assumption that both labelled and unlabelled UMP would be incorporated with the same efficiency. Sample calculations follow.

Figure 2.2 CPM vs. pmole ^{3}H-UTP


18
14
10
6
2
CPM (X 10⁻³)
Slope = 19 794 CPM p mole ³H-UTP
.2
.4
.6
.8
1.0
³H-UTP (p mole)

Five μCi (111 pmoles) of ^{3}H-UTP were normally present in an assay mixture. The incorporation of this amount of ^{3}H-UTP would result in 2,197,134 CPM (i.e. 19,794 CPM/pmole ^{3}H-UTP x 111 pmoles ^{3}H-UTP). The amount of total UTP present in the assay was 10,111 picomoles (i.e. 10,000 pmoles unlabelled UTP + 111 pmoles labelled UTP). A count of 217 CPM (i.e. 2,197,134 CPM ÷ 10,111 pmoles) was therefore considered to represent, for this batch of ^{3}H-UTP, the incorporation of one picomole UMP.

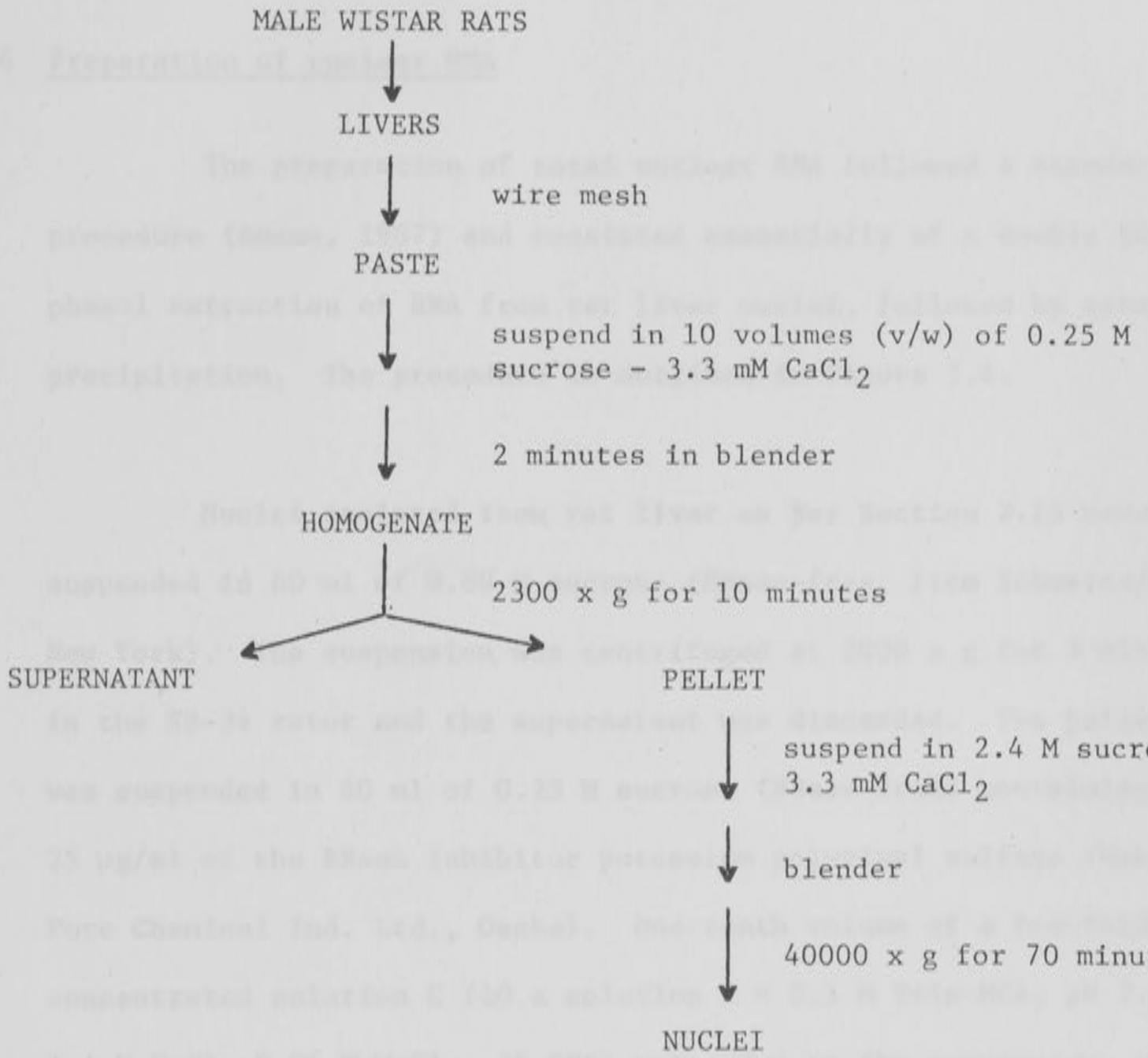
These calculations were carried out for each batch of ^{3}H-NTP utilized.

2.15 Isolation of nuclei from rat livers

The procedure used in the isolation of nuclei from rat livers is outlined in Figure 2.3.

Seven male Wistar rats (160-180 g) were sacrificed and the livers removed. These were passed through a wire mesh (354 μ) and the paste was suspended in 10 volumes (v/w) of 0.25 M sucrose - 3.3 mM $CaCl_2$. The paste, with its "hardened" nuclei, was subjected to a thorough blending (Universal Homogenizer, Nihon Seiki Seisakusho Co., Tokyo). The homogenate was centrifuged at 2300 x g for 10 minutes in the Sorvall RC2-B centrifuge with the GSA rotor. The supernatant was decanted and the pellet was suspended in about 200 ml of 2.4 M sucrose - 3.3 mM $CaCl_2$. This suspension was thoroughly stirred in the blender and then centrifuged at 40,000 x g for 70 minutes in the Model L centrifuge using the 21 rotor. An essentially pure preparation of nuclei, as judged by microscopic examination (Nikon

Figure 2.3 Isolation of nuclei from rat livers.


MALE WISTAR RATS
LIVERS
wire mesh
PASTE
suspend in 10 volumes (v/w) of 0.25 M sucrose - 3.3 mM $CaCl_2$
2 minutes in blender
HOMOGENATE
2300 x g for 10 minutes
SUPERNATANT
PELLET
suspend in 2.4 M sucrose - 3.3 mM $CaCl_2$
blender
40000 x g for 70 minutes
NUCLEI

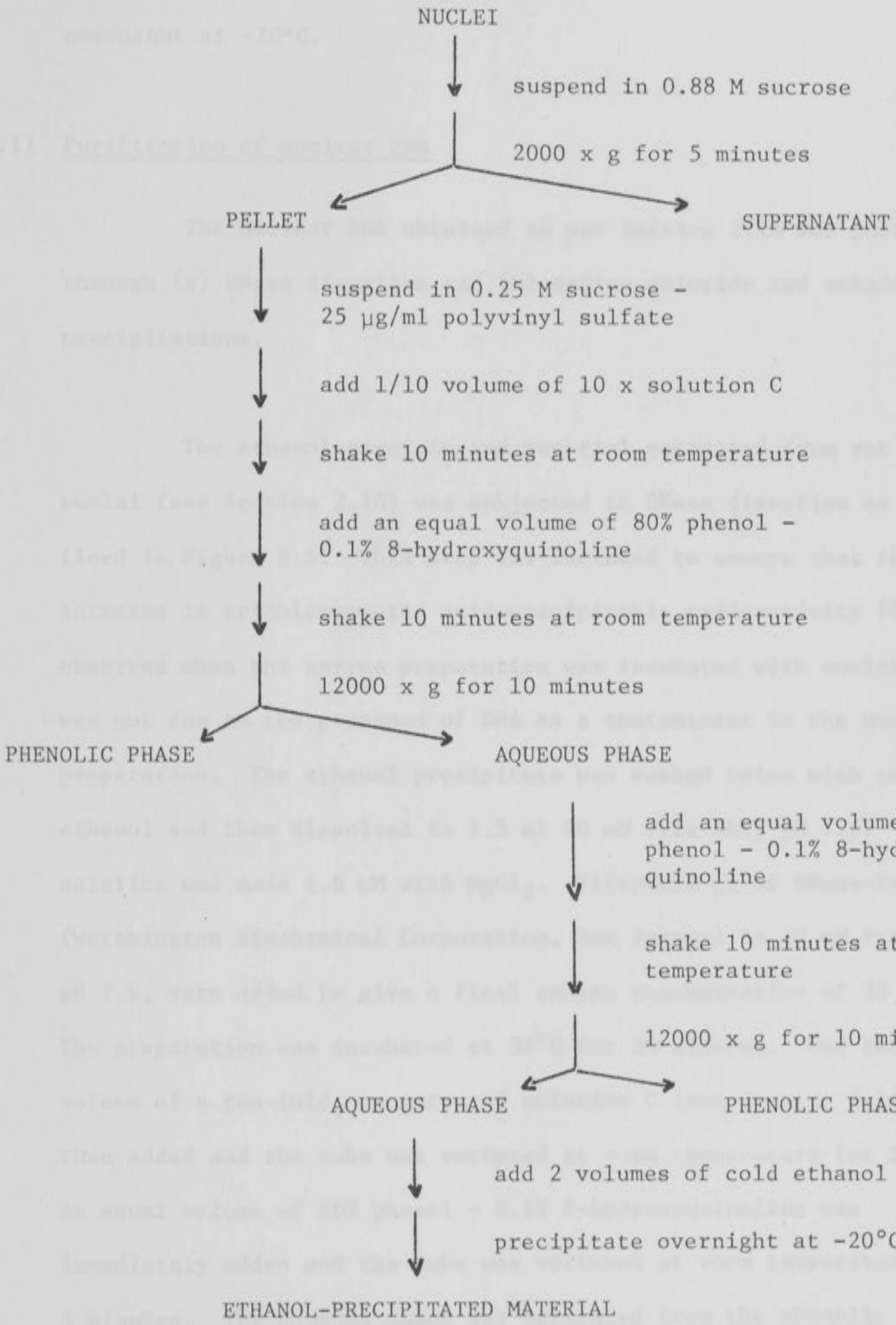
microscope model S-Ke II from Nippon Kogaku K.K., Tokyo) was recovered as a white pellet.

2.16 Preparation of nuclear RNA

The preparation of total nuclear RNA followed a standard procedure (Amano, 1967) and consisted essentially of a double SDS-phenol extraction of RNA from rat liver nuclei, followed by ethanol precipitation. The procedure is outlined in Figure 2.4.

Nuclei isolated from rat liver as per Section 2.15 were suspended in 80 ml of 0.88 M sucrose (RNase-free, from Schwartz/Mann, New York). The suspension was centrifuged at 2000 x g for 5 minutes in the SS-34 rotor and the supernatant was discarded. The pellet was suspended in 60 ml of 0.25 M sucrose (RNase-free) containing 25 µg/ml of the RNase inhibitor potassium polyvinyl sulfate (Wako Pure Chemical Ind. Ltd., Osaka). One tenth volume of a ten-fold concentrated solution C (10 x solution C = 0.1 M Tris-HCl, pH 7.6, 1.4 M NaCl, 0.05 M $MgCl_2$, 2% SDS) was added to the suspension, which was then shaken at room temperature for 10 minutes. An equal volume of 80% phenol (Wako, Osaka) - 0.1% 8-hydroxyquinoline (B.D.H. Chemicals Ltd., England) was then added and the suspension was again shaken at room temperature for 10 minutes. The phenolic and aqueous phases were separated by centrifugation at 12000 x g for 10 minutes in the SS-34. The aqueous phase, containing the RNA, was recovered. An equal volume of 80% phenol - 0.1% 8-hydroxyquinoline was added to it and the shaking for 10 minutes at room temperature was repeated. The aqueous phase was separated from the phenolic phase and recovered as before. Two volumes of cold ethanol (Merck, Darmstadt) were

Figure 2.4 Preparation of nuclear RNA: extraction of RNA from nuclei and ethanol precipitation.


NUCLEI
suspend in 0.88 M sucrose
2000 x g for 5 minutes
PELLET
SUPERNATANT
suspend in 0.25 M sucrose - 25 µg/ml polyvinyl sulfate
add 1/10 volume of 10 x solution C
shake 10 minutes at room temperature
add an equal volume of 80% phenol - 0.1% 8-hydroxyquinoline
shake 10 minutes at room temperature
12000 x g for 10 minutes
PHENOLIC PHASE
AQUEOUS PHASE
add an equal volume of 80% phenol - 0.1% 8-hydroxy-quinoline
shake 10 minutes at room temperature
12000 x g for 10 minutes
AQUEOUS PHASE
PHENOLIC PHASE
add 2 volumes of cold ethanol
precipitate overnight at -20°C
ETHANOL-PRECIPITATED MATERIAL

added to the aqueous phase. The RNA was allowed to precipitate overnight at -20°C.

2.17 Purification of nuclear RNA

The nuclear RNA obtained as per Section 2.16 was purified through (a) DNase digestion and (b) sodium chloride and ethanol precipitations.

The ethanol-precipitated material extracted from rat liver nuclei (see Section 2.16) was subjected to DNase digestion as outlined in Figure 2.5. This step was included to ensure that the increase in trichloroacetic acid-precipitable radioactivity (CPM) observed when the enzyme preparation was incubated with nuclear RNA was not due to the presence of DNA as a contaminant in the nuclear RNA preparation. The ethanol precipitate was washed twice with cold 80% ethanol and then dissolved in 1.5 ml 10 mM Tris-HCl, pH 7.6. The solution was made 1.6 mM with $MgCl_2$. Fifty-six µg of RNase-free DNase (Worthington Biochemical Corporation, New Jersey) in 10 mM Tris-HCl, pH 7.6, were added to give a final enzyme concentration of 35 µg/ml. The preparation was incubated at 37°C for 20 minutes. One tenth volume of a ten-fold concentrated solution C (see Section 2.16) was then added and the tube was vortexed at room temperature for 10 minutes. An equal volume of 80% phenol - 0.1% 8-hydroxyquinoline was immediately added and the tube was vortexed at room temperature for 5 minutes. The aqueous phase was separated from the phenolic phase by centrifugation at 12000 x g for 10 minutes in the SS-34 rotor. The aqueous phase was recovered. Two volumes of cold ethanol were added and the nuclear RNA was allowed to precipitate overnight at -20°C.

Figure 2.5 DNase digestion of nuclear RNA

ETHANOL-PRECIPITATED MATERIAL

↓ 80% ethanol washes

↓ dissolve in 10 mM Tris-HCl, pH 7.6

↓ add $MgCl_2$

↓ add DNase and incubate (37°C for 20 minutes)

↓ add 1/10 volume of 10 x solution C and vortex

↓ add an equal volume of 80% phenol - 0.1% 8-hydroxyquinoline and vortex

↓ 12000 x g for 10 minutes

PHENOLIC PHASE

AQUEOUS PHASE

↓ add 2 volumes of cold ethanol and precipitate overnight at -20°C

nuclear RNA

The final steps in the purification of nuclear RNA are outlined in Figure 2.6. The ethanol-precipitated RNA was collected using the Sorvall GLC-1 centrifuge (HL-4 rotor) at 1000 x g for 5 minutes. It was dissolved in 2 ml of 10 mM Tris-HCl, pH 7.6. Sodium chloride was added to a final concentration of 0.1 M. Two volumes of cold ethanol were then added and the nuclear RNA was allowed to precipitate for 3 hours at -20°C. The RNA was collected, redissolved and reprecipitated once more in the manner described above. The final precipitate was collected, washed in cold 80% ethanol and dissolved in 0.8 ml of 10 mM Tris-HCl, pH 7.6. The solution was centrifuged at 12000 x g for 15 minutes in the SS-34 rotor. The supernatant was collected and stored at -20°C. A typical preparation, starting with 7 rats, yielded approximately 3 mg of nuclear RNA.

2.18 Preparation of whole cell RNA from rat livers

Whole cell RNA from rat livers was used as carrier throughout the experiments. The method of preparation (Monckton and Naora, 1974) is described in this section and outlined in Figure 2.7. Three male Wistar rats (160-180 g) were starved overnight and their livers were removed. The livers (16 g wet weight) were passed through a wire mesh (354 μ). Ten volumes (v/w) of 0.25 M sucrose were added and the livers were homogenized (Universal Homogenizer from Nihon Seiki Seisakusho, Tokyo). One tenth volume of ten-fold concentrated solution C (see Section 2.16) was added to the homogenate and the mixture was shaken for 10 minutes. An equal volume of 80% phenol - 0.1% 8-hydroxy-quinoline was then added and the shaking repeated for another 10 minutes. The aqueous phase was separated from the phenolic phase by a 10 minute centrifugation at 24000 x g. An equal volume of 80%

Figure 2.6 Sodium chloride and ethanol precipitations of nuclear RNA.

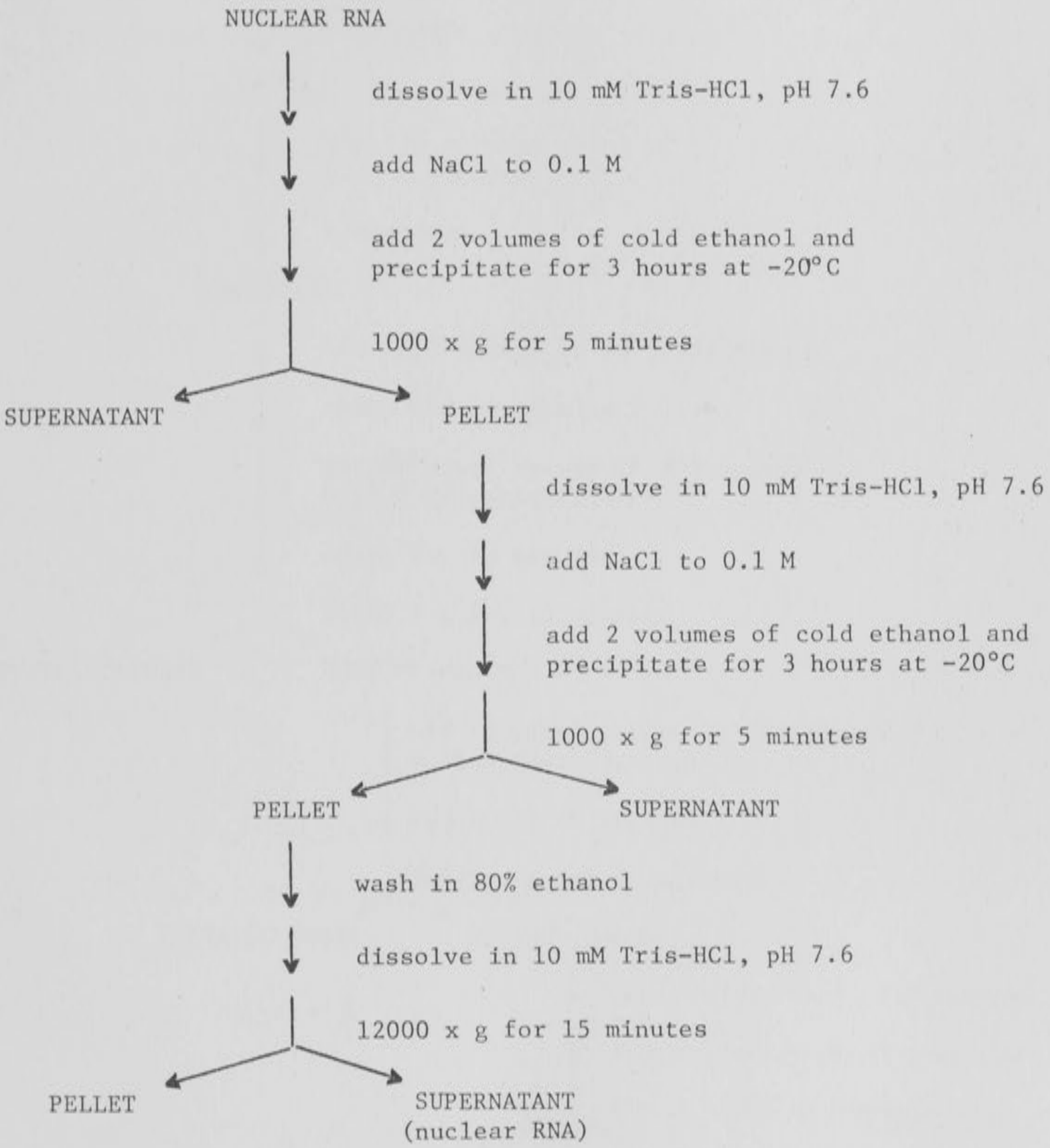
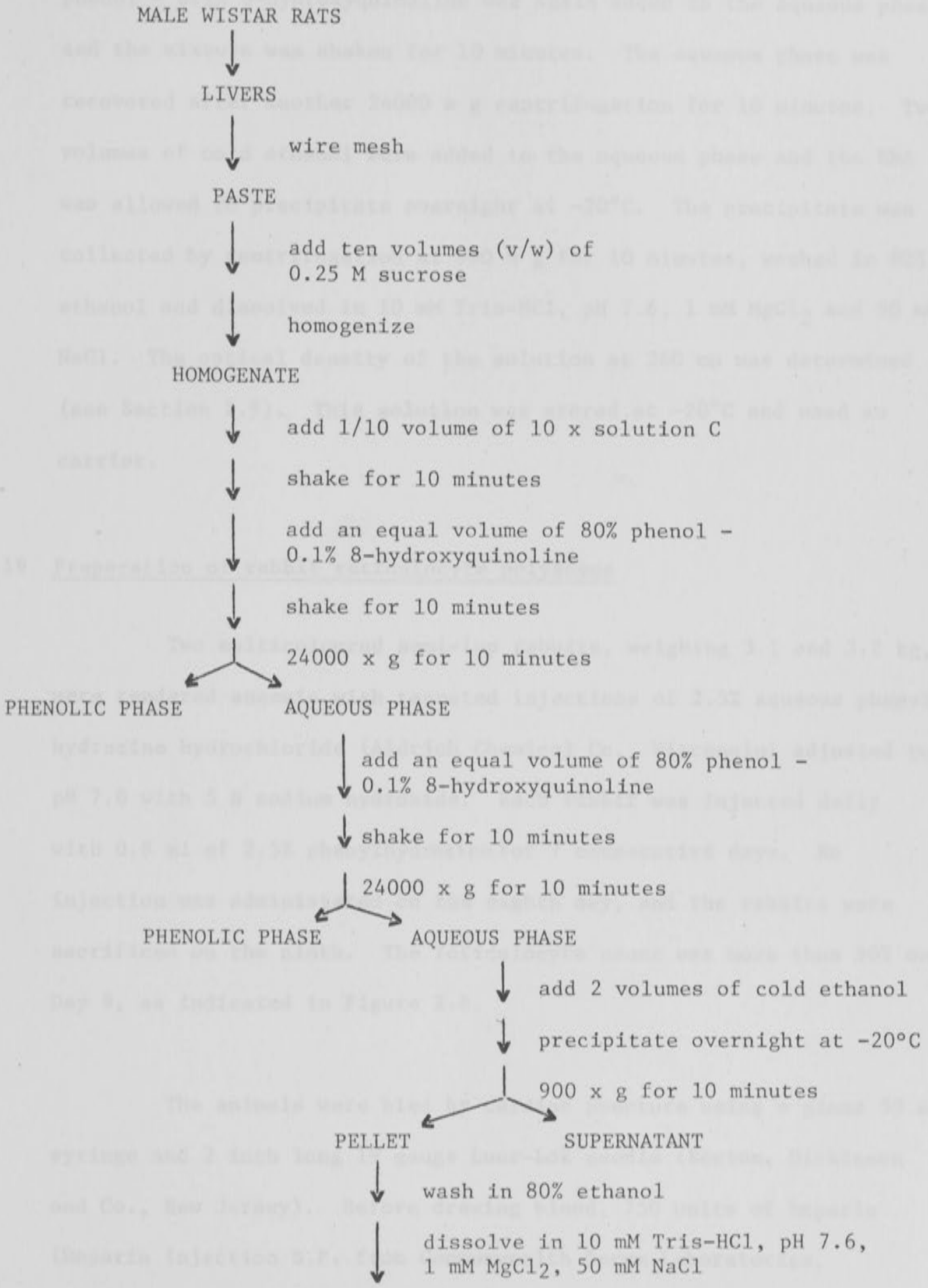

NUCLEAR RNA
dissolve in 10 mM Tris-HCl, pH 7.6
add NaCl to 0.1 M
add 2 volumes of cold ethanol and precipitate for 3 hours at -20°C
1000 x g for 5 minutes
SUPERNATANT
PELLET
dissolve in 10 mM Tris-HCl, pH 7.6
add NaCl to 0.1 M
add 2 volumes of cold ethanol and precipitate for 3 hours at -20°C
1000 x g for 5 minutes
PELLET
SUPERNATANT
wash in 80% ethanol
dissolve in 10 mM Tris-HCl, pH 7.6
12000 x g for 15 minutes
PELLET
SUPERNATANT
(nuclear RNA)

Figure 2.7 Preparation of whole cell RNA from rat liver.


MALE WISTAR RATS
LIVERS
wire mesh
PASTE
add ten volumes (v/w) of
0.25 M sucrose
homogenize
HOMOGENATE
add 1/10 volume of 10 x solution C
shake for 10 minutes
add an equal volume of 80% phenol -
0.1% 8-hydroxyquinoline
shake for 10 minutes
24000 x g for 10 minutes
PHENOLIC PHASE
AQUEOUS PHASE
add an equal volume of 80% phenol -
0.1% 8-hydroxyquinoline
shake for 10 minutes
24000 x g for 10 minutes
PHENOLIC PHASE
AQUEOUS PHASE
add 2 volumes of cold ethanol
precipitate overnight at -20°C
900 x g for 10 minutes
PELLET
SUPERNATANT
wash in 80% ethanol
dissolve in 10 mM Tris-HCl, pH 7.6,
1 mM MgCl2, 50 mM NaCl
CARRIER RNA

phenol - 0.1% 8-hydroxyquinoline was again added to the aqueous phase and the mixture was shaken for 10 minutes. The aqueous phase was recovered after another 24000 x g centrifugation for 10 minutes. Two volumes of cold ethanol were added to the aqueous phase and the RNA was allowed to precipitate overnight at -20°C. The precipitate was collected by centrifugation at 900 x g for 10 minutes, washed in 80% ethanol and dissolved in 10 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$ and 50 mM NaCl. The optical density of the solution at 260 mμ was determined (see Section 2.9). This solution was stored at -20°C and used as carrier.

2.19 Preparation of rabbit reticulocyte polysomes

Two multicoloured semi-lop rabbits, weighing 3.1 and 3.2 kg, were rendered anaemic with repeated injections of 2.5% aqueous phenylhydrazine hydrochloride (Aldrich Chemical Co., Wisconsin) adjusted to pH 7.0 with 5 N sodium hydroxide. Each rabbit was injected daily with 0.8 ml of 2.5% phenylhydrazine for 7 consecutive days. No injection was administered on the eighth day, and the rabbits were sacrificed on the ninth. The reticulocyte count was more than 90% on Day 9, as indicated in Figure 2.8.

The animals were bled by cardiac puncture using a glass 50 ml syringe and 2 inch long 19 gauge Luer-Lok needle (Becton, Dickinson and Co., New Jersey). Before drawing blood, 750 units of heparin (Heparin Injection B.P. from Commonwealth Serum Laboratories, Melbourne) in 0.14 M NaCl were added to each syringe. The remainder of the preparation was based on the method of Schreier and Staehelin (1973). It is outlined in Figure 2.9.

Figure 2.8 Induction of reticulocytosis. Rabbits were rendered anaemic as described in the text. Day 1 was the day of the first injection.

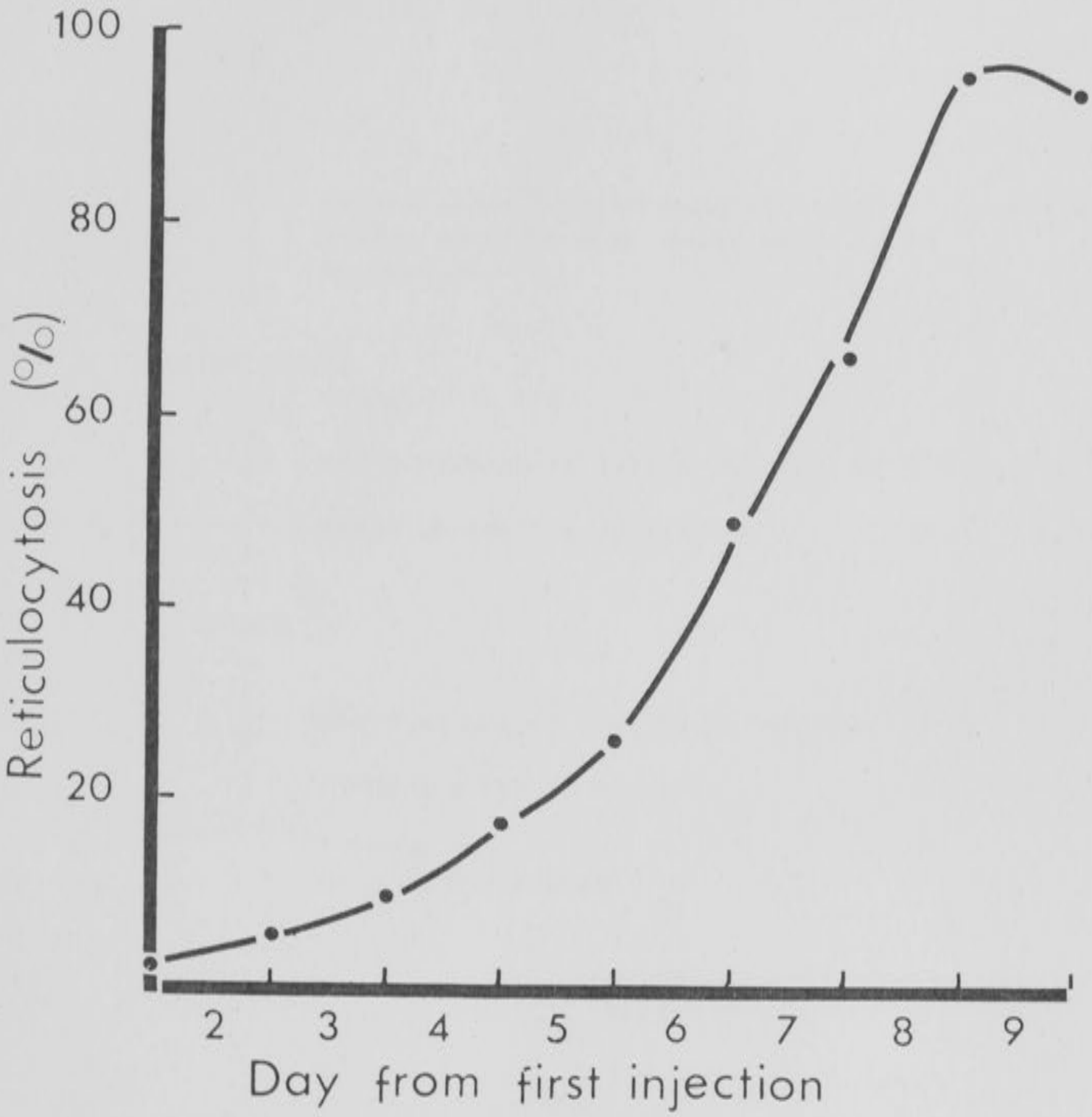

100
80
60
40
20
Reticulocytosis (%)
2
3
4
5
6
7
8
9
Day from first injection

Figure 2.9 Preparation of rabbit reticulocyte polysomes.

BLOOD

↓ strain through 3 layers of cheesecloth

↓ 500 x g for 10 minutes

CELLS

↓ wash 5 times in 4 volumes of isotonic saline solution and remove white cells by suction

RETICULOCYTES

↓ add 4 volumes of lyzing solution at 0°C

↓ stand on ice for 30 seconds

LYSATE

↓ add 1 volume of post-lysis solution

↓ 15000 x g for 10 minutes

PELLET ← → SUPERNATANT

↓ layer over 36% sucrose in Buffer P

↓ 70000 x g for 3 hours

SUPERNATANT ← → POLYSOMES

The blood was strained through 3 layers of cheesecloth to eliminate clots. The cells were pelleted with a 500 x g centrifugation for 10 minutes. They were then washed 5 times in 4 volumes of isotonic saline solution (130 mM NaCl, 7.4 mM $MgCl_2$, 5 mM KCl) and the white cells were removed by suction. Four volumes of lyzing solution (4 mM $MgCl_2$) were added to the reticulocytes and the cell suspension was left on ice for 30 seconds. One volume (volume of packed cells, before the addition of lyzing solution) of post-lysis solution (0.15 M KCl, 1.5 M sucrose) was then added to the lysate and it was centrifuged at 15000 x g for 10 minutes. The supernatant was layered over 36% sucrose in Buffer P (10 mM Tris-HCl, pH 7.4, 15 mM KCl, 5 mM magnesium acetate). After a 3 hour centrifugation at 70000 x g, the polysomes were recovered as a pellet at the bottom of the tubes.

2.20 Preparation of rabbit reticulocyte "9S" RNA

Rabbit reticulocyte polysomes (Section 2.19) were homogenized (Thomas tissue grinder, Philadelphia) in 10 mM Tris-HCl, pH 7.5, with a loose-fitting Teflon-tipped pestle using 5 strokes at setting 1 on the Caframo (Ontario, Canada) RZR1-64 stirrer. The homogenate was centrifuged at 15000 x g for 5 minutes. The optical density of the supernatant at 260 mμ was determined (Section 2.9). The supernatant was then diluted in 10 mM Tris-HCl, pH 7.5, to 25 OD_{260}/ml. RNA was then extracted as per Schreier and Staehelin (1973).

The ethanol-precipitated RNA was collected by centrifugation (900 x g for 10 minutes) and washed twice in 80% ethanol. It was

then dissolved in 10 mM Tris-HCl, pH 7.5, and the OD_{260} was determined.

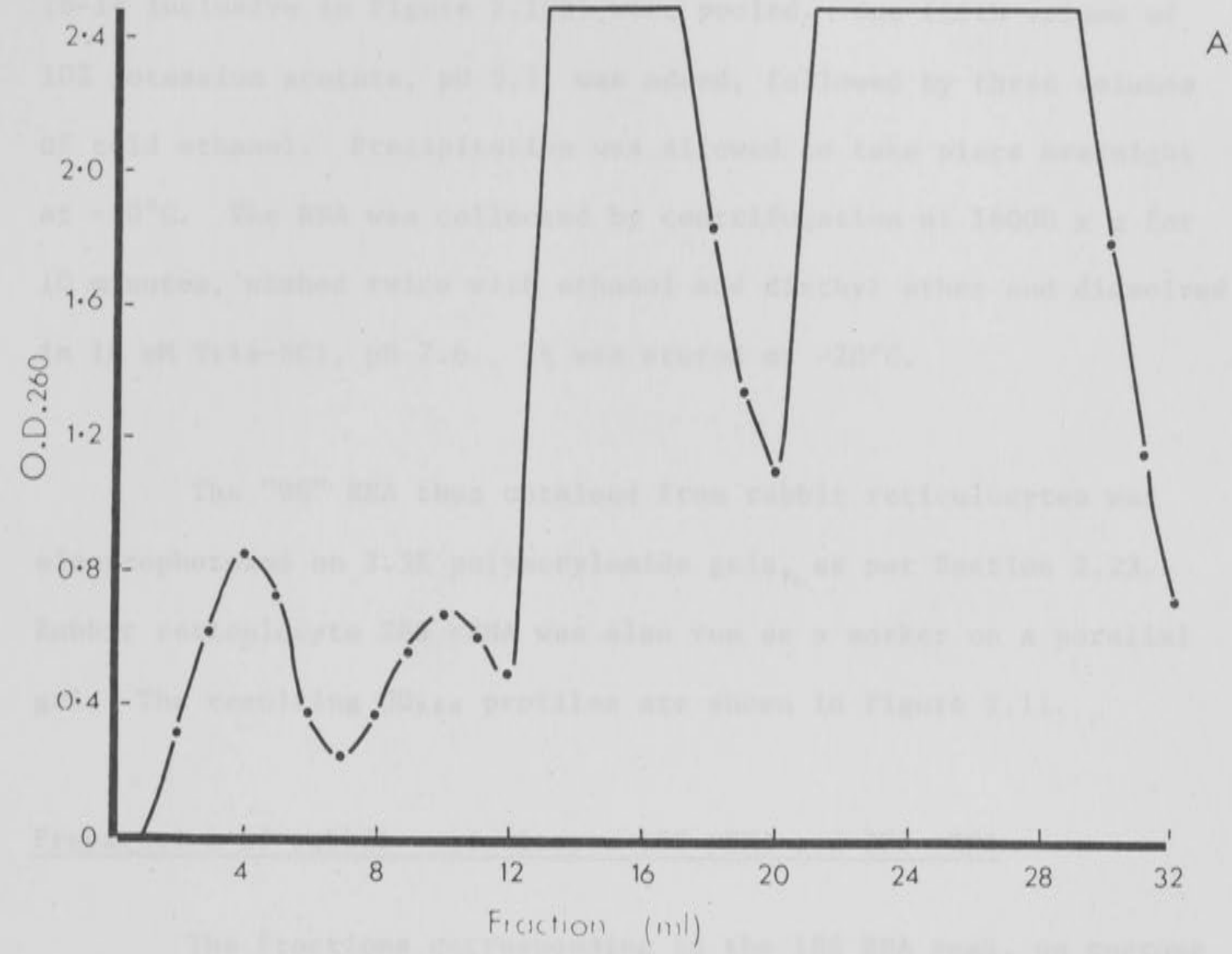
Approximately 100 OD_{260} units were layered onto each of two 35 ml linear sucrose gradients (5-30% sucrose in 10 mM Tris-HCl, pH 7.6, 0.14 M NaCl) prepared with RNase-free sucrose (Schwartz/ Mann, New York) using an Isco (Lincoln, Nebraska) Model 570 gradient former. The sucrose gradients were centrifuged for 18 hours at 112700 x g.

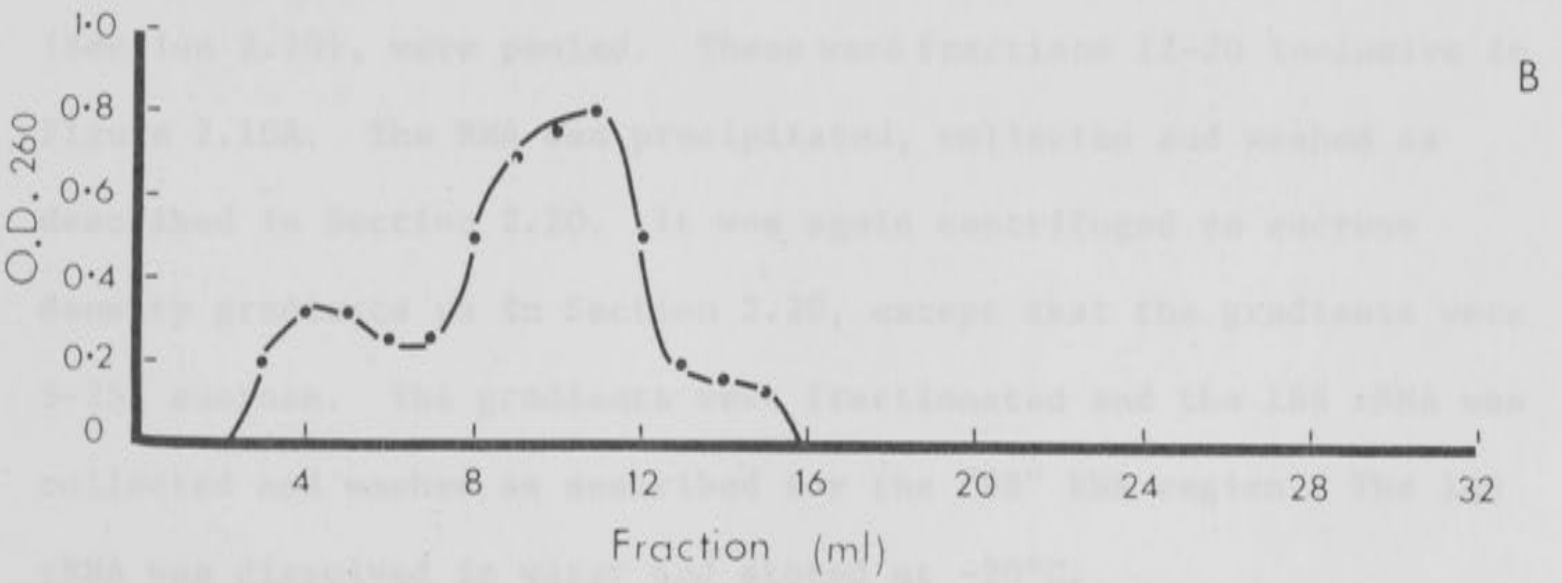
The gradients were fractionated using an Isco Model D density gradient fractionator in conjunction with an Isco UA-2 ultraviolet analyzer with a 10 mm (light path length) flow cell. One ml fractions were collected. A typical OD_{260} profile is shown in Figure 2.10A.

The OD_{260} peak corresponding to the "9S" RNA (fractions 6-11 inclusive in Figure 2.10A) was collected from the gradients and pooled. One fifth volume of 10% potassium acetate (B.D.H. Chemicals Ltd., England), pH 5.3, was added. Three volumes of cold ethanol were then added and precipitation was allowed to take place overnight at -20°C.

The RNA was collected by centrifugation at 16000 x g for 20 minutes. The pellet was dissolved in 10 mM Tris-HCl, pH 7.5, and layered onto sucrose gradients as before. The gradients were again centrifuged at 112700 x g for 18 hours and fractionated as before. The OD_{260} profile resulting from the second centrifugation is shown

Figure 2.10 Sucrose density gradient centrifugation of polysomal RNA (A) and of the "9S" region (B) obtained from the first centrifugation. Gradients were from 5-30% sucrose and centrifugation was for 18 hours at 112700 x g.


A
O.D.260
2·4
2·0
1·6
1·2
0·8
0·4
0
4
8
12
16
20
24
28
32
Fraction (ml)


B
O.D. 260
1·0
0·8
0·6
0·4
0·2
0
4
8
12
16
20
24
28
32
Fraction (ml)

in Figure 2.10B. The fractions corresponding to the "9S" RNA peak (8-14 inclusive in Figure 2.10B) were pooled. One fifth volume of 10% potassium acetate, pH 5.3, was added, followed by three volumes of cold ethanol. Precipitation was allowed to take place overnight at -20°C. The RNA was collected by centrifugation at 16000 x g for 10 minutes, washed twice with ethanol and diethyl ether and dissolved in 10 mM Tris-HCl, pH 7.6. It was stored at -20°C.

The "9S" RNA thus obtained from rabbit reticulocytes was electrophorezed on 2.5% polyacrylamide gels, as per Section 2.23. Rabbit reticulocyte 28S rRNA was also run as a marker on a parallel gel. The resulting OD_{260} profiles are shown in Figure 2.11.

2.21 Preparation of rabbit reticulocyte 18S rRNA and 28S rRNA

The fractions corresponding to the 18S RNA peak, on sucrose density gradient centrifugation of rabbit reticulocyte polysomal RNA (Section 2.20), were pooled. These were fractions 12-20 inclusive in Figure 2.10A. The RNA was precipitated, collected and washed as described in Section 2.20. It was again centrifuged on sucrose density gradients as in Section 2.20, except that the gradients were 5-25% sucrose. The gradients were fractionated and the 18S rRNA was collected and washed as described for the "9S" RNA region. The 18S rRNA was dissolved in water and stored at -20°C.

The fractions corresponding to the 28S rRNA peak (fractions 21-31 inclusive in Figure 2.10A), on sucrose density gradient centrifugation of rabbit reticulocyte polysomal RNA, were pooled and were treated as above.

Figure 2.11 PAG (2.5%) electrophoresis of the "9S" RNA region obtained after 2 sucrose density gradient (5-30%) centrifugations at 112700 x g for 18 hours (fractions 8-14 inclusive in Figure 2.10B). The dashed line indicates the location of 28S rRNA from rabbit reticulocytes which was run as a marker on a parallel gel. Electrophoresis was for 80 minutes at 7 mA/gel.

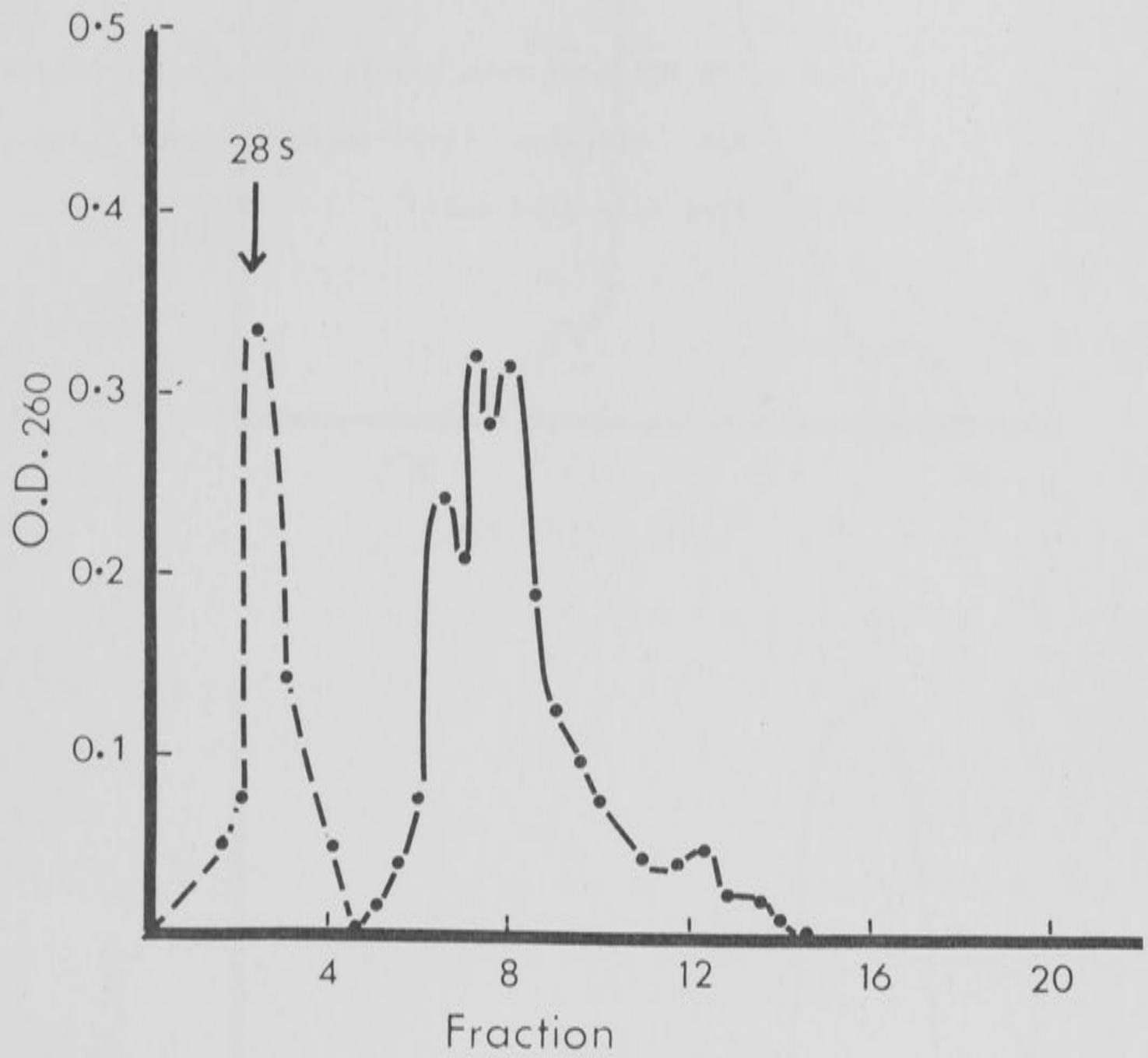
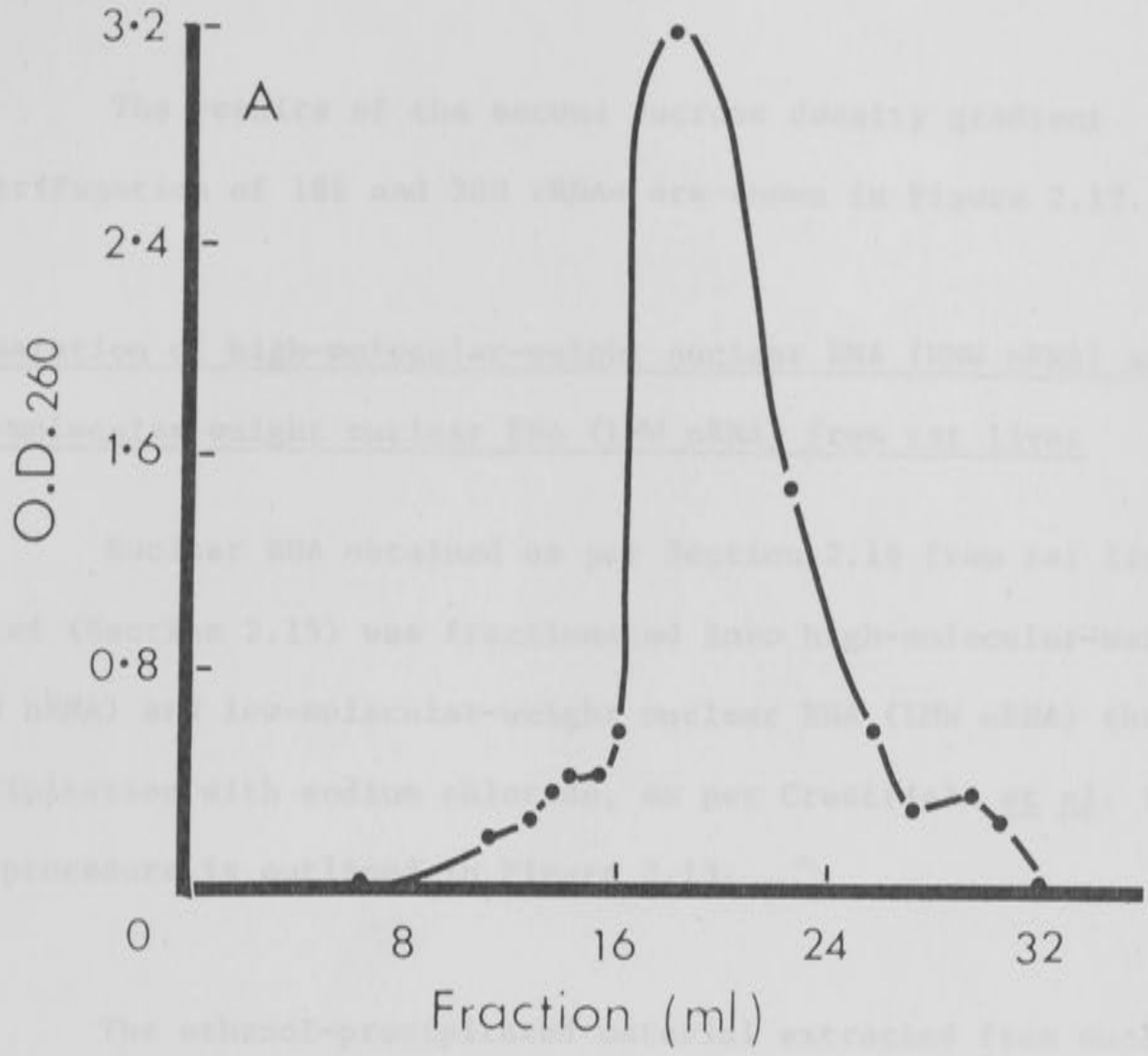
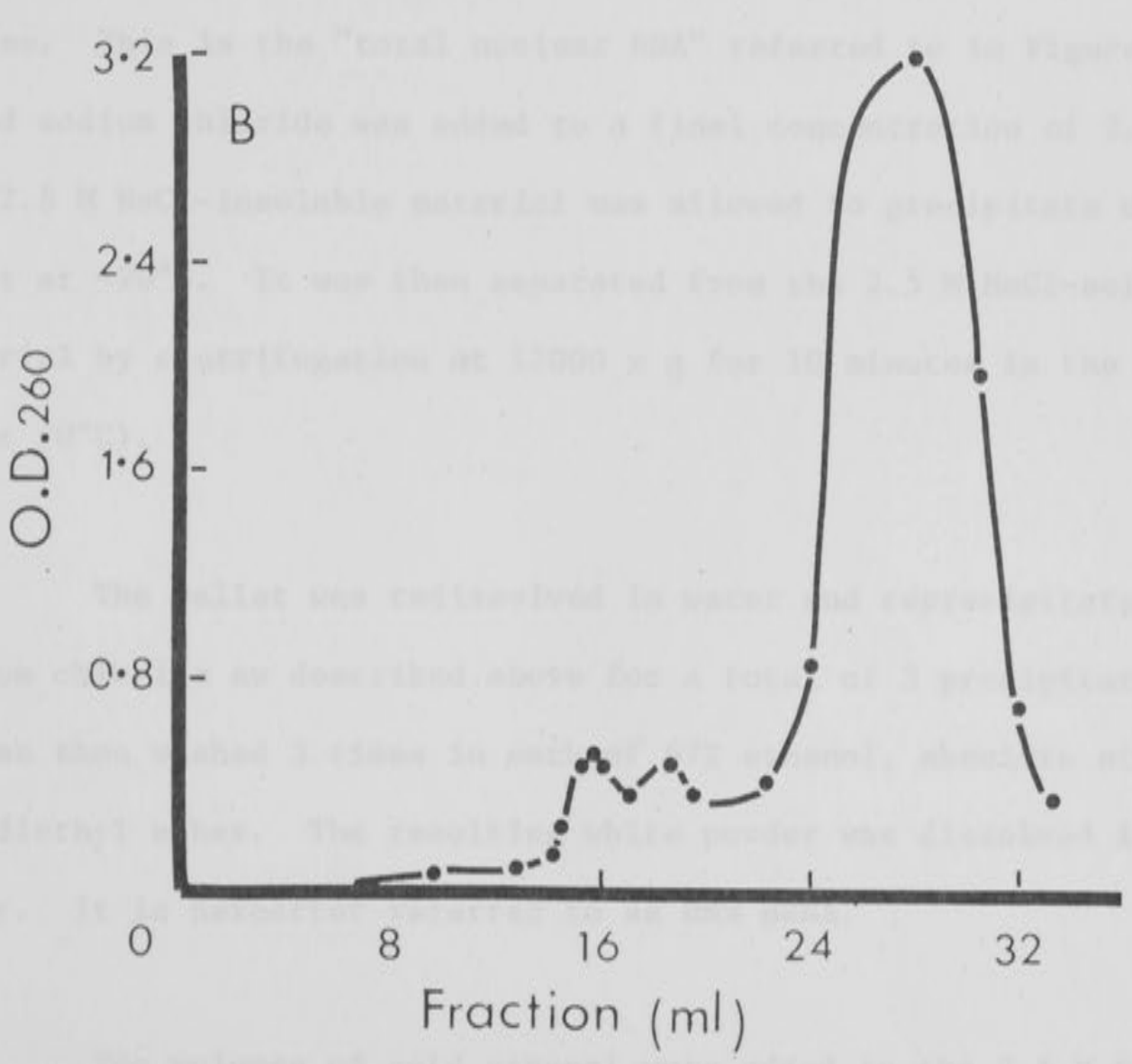

0·5
0·4
0·3
0·2
0.1
O.D. 260
28 s
4
8
12
16
20
Fraction

Figure 2.12 Sucrose density gradient centrifugation of

A - 18S RNA pool from rabbit reticulocyte polysomal RNA. Fractions 16-23 inclusive were collected from this gradient.

B - 28S RNA pool from rabbit reticulocyte polysomal RNA. Fractions 24-32 inclusive were collected from this gradient.


A
3·2
2·4
1·6
0·8
0
O.D. 260
8
16
24
32
Fraction (ml)


B
3·2
2·4
1·6
0·8
0
O.D.260
8
16
24
32
Fraction (ml)

The results of the second sucrose density gradient centrifugation of 18S and 28S rRNAs are shown in Figure 2.12.

2.22 Preparation of high-molecular-weight nuclear RNA (HMW nRNA) and low-molecular-weight nuclear RNA (LMW nRNA) from rat liver

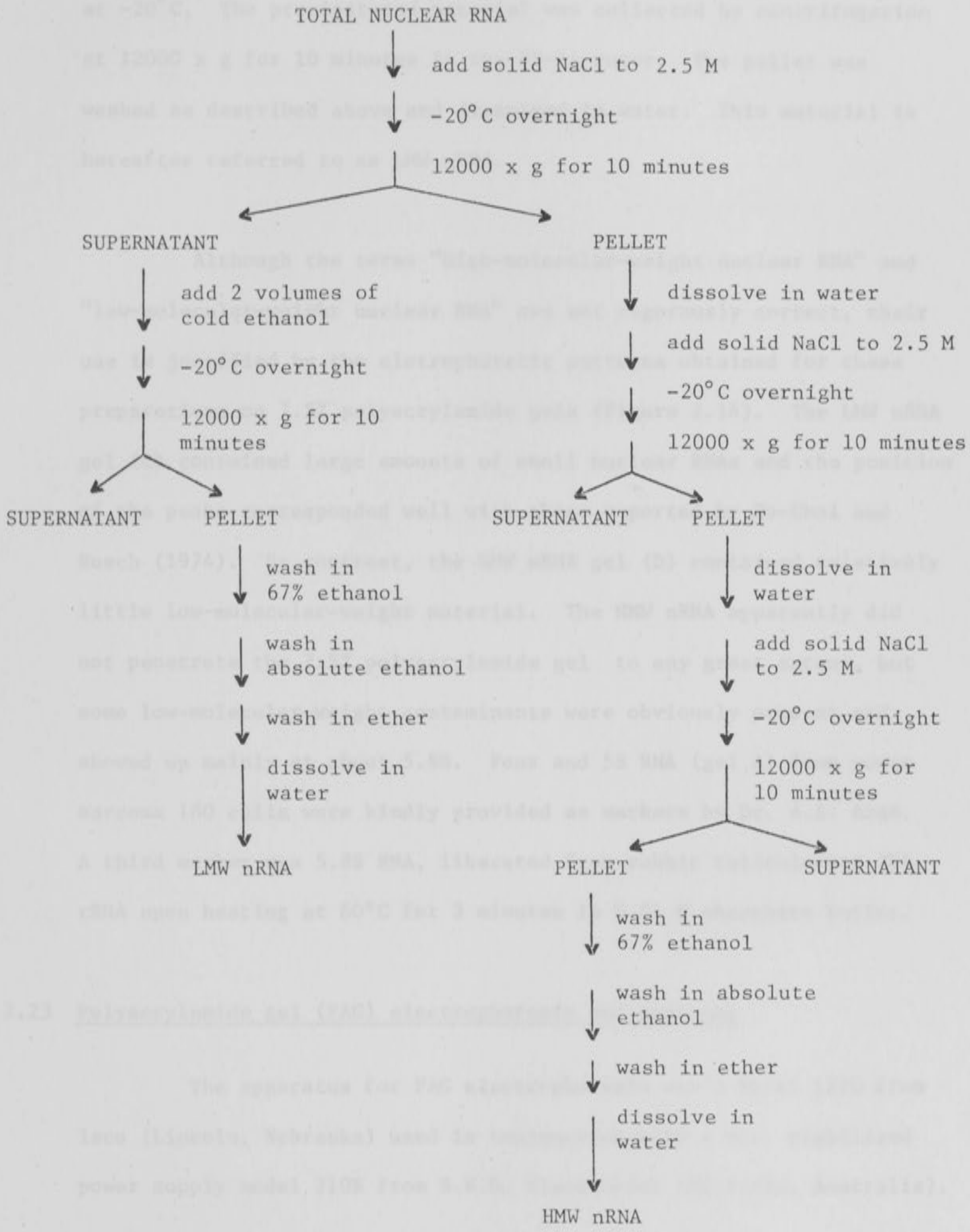
Nuclear RNA obtained as per Section 2.16 from rat liver nuclei (Section 2.15) was fractionated into high-molecular-weight (HMW nRNA) and low-molecular-weight nuclear RNA (LMW nRNA) through precipitation with sodium chloride, as per Crestfield *et al*. (1955). The procedure is outlined in Figure 2.13.

The ethanol-precipitated material extracted from nuclei (Figure 2.4) was dissolved in 2 ml of water after several 80% ethanol washes. This is the "total nuclear RNA" referred to in Figure 2.13. Solid sodium chloride was added to a final concentration of 2.5 M. The 2.5 M NaCl-insoluble material was allowed to precipitate overnight at -20°C. It was then separated from the 2.5 M NaCl-soluble material by centrifugation at 12000 x g for 10 minutes in the SS-34 rotor (0°C).

The pellet was redissolved in water and reprecipitated with sodium chloride as described above for a total of 3 precipitations. It was then washed 3 times in each of 67% ethanol, absolute ethanol and diethyl ether. The resulting white powder was dissolved in water. It is hereafter referred to as HMW nRNA.

Two volumes of cold ethanol were added to the 2.5 M NaCl-soluble fraction and precipitation was allowed to take place overnight

Figure 2.13 Preparation of HMW nRNA and LMW nRNA.


TOTAL NUCLEAR RNA
add solid NaCl to 2.5 M
-20°C overnight
12000 x g for 10 minutes
SUPERNATANT
add 2 volumes of cold ethanol
-20°C overnight
12000 x g for 10 minutes
SUPERNATANT
PELLET
wash in 67% ethanol
wash in absolute ethanol
wash in ether
dissolve in water
LMW nRNA
PELLET
dissolve in water
add solid NaCl to 2.5 M
-20°C overnight
12000 x g for 10 minutes
SUPERNATANT
PELLET
dissolve in water
add solid NaCl to 2.5 M
-20°C overnight
12000 x g for 10 minutes
PELLET
SUPERNATANT
wash in 67% ethanol
wash in absolute ethanol
wash in ether
dissolve in water
HMW nRNA

at -20°C. The precipitated material was collected by centrifugation at 12000 x g for 10 minutes in the SS-34 rotor. The pellet was washed as described above and dissolved in water. This material is hereafter referred to as LMW nRNA.

Although the terms "high-molecular-weight nuclear RNA" and "low-molecular-weight nuclear RNA" are not rigorously correct, their use is justified by the eletrophoretic patterns obtained for these preparations on 7.5% polyacrylamide gels (Figure 2.14). The LMW nRNA gel (C) contained large amounts of small nuclear RNAs and the position of the peaks corresponded well with those reported by Ro-Choi and Busch (1974). By contrast, the HMW nRNA gel (D) contained relatively little low-molecular-weight material. The HMW nRNA apparently did not penetrate the 7.5% polyacrylamide gel to any great extent, but some low-molecular-weight contaminants were obviously present and showed up mainly at about 5.8S. Four and 5S RNA (gel A) from mouse sarcoma 180 cells were kindly provided as markers by Dr. A.A. Azad. A third marker was 5.8S RNA, liberated from rabbit reticulocyte 28S rRNA upon heating at 60°C for 3 minutes in 0.01 M phosphate buffer.

2.23 Polyacrylamide gel (PAG) electrophoresis and scanning

The apparatus for PAG electrophoresis was a Model 1270 from Isco (Lincoln, Nebraska) used in conjunction with a D.C. stabilized power supply model 210B from B.W.D. Electronics (Victoria, Australia).

Acrylamide, N,N'-methylene-bis-acrylamide, ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED) catalyst were obtained as a kit from BIO-RAD Laboratories (California).

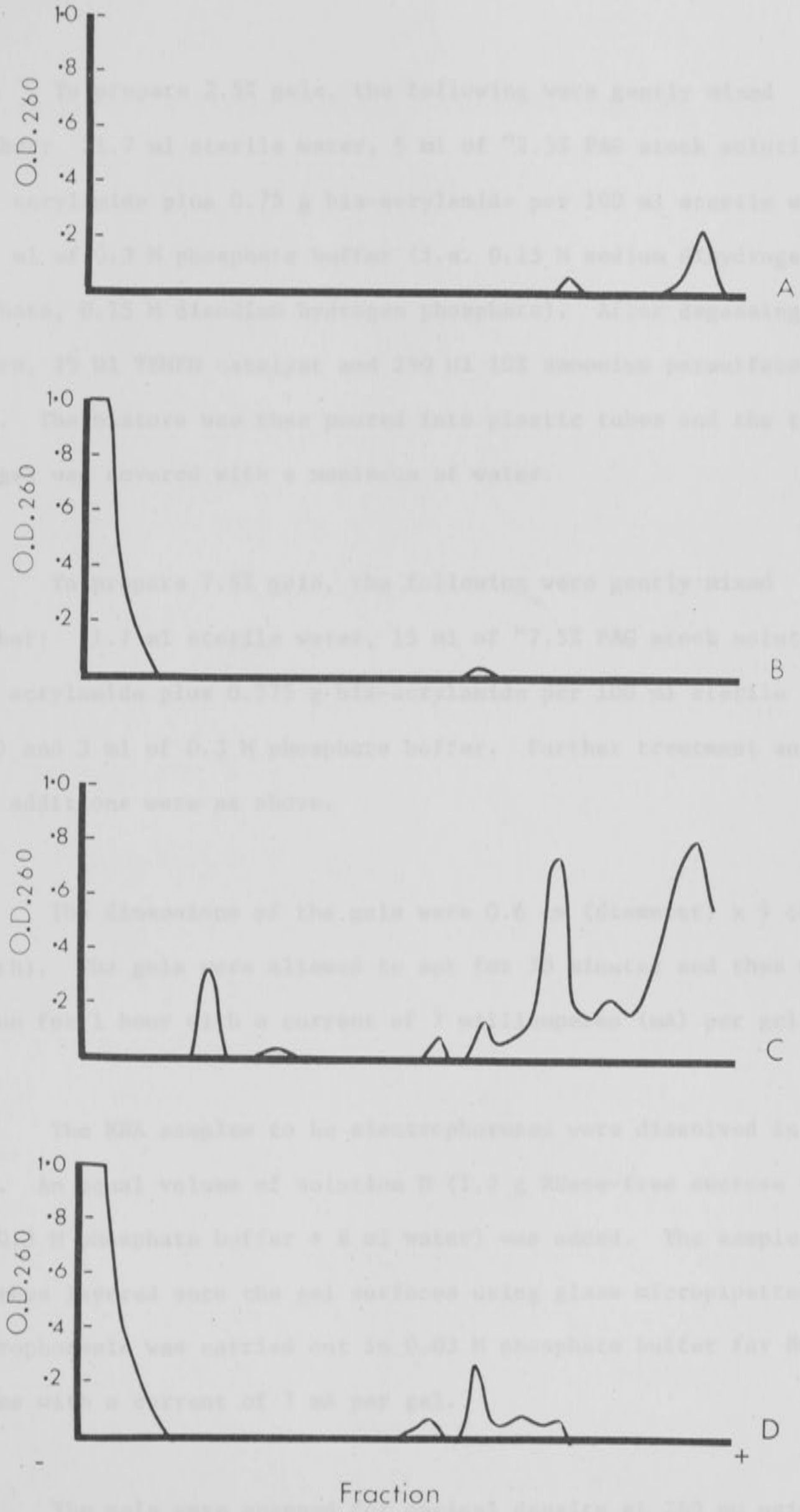
Figure 2.14 Polyacrylamide gel electrophoresis (7.5%) of

(A) 4 and 5S RNAs from mouse sarcoma 180 cells

(B) heated 28S rRNA from rabbit reticulocytes

(C) LMW nRNA from rat livers

(D) HMW nRNA from rat livers


1·0
·8
·6
·4
·2
O.D.260
A
B
C
D
-
+
Fraction

To prepare 2.5% gels, the following were gently mixed together: 21.7 ml sterile water, 5 ml of "2.5% PAG stock solution" (15 g acrylamide plus 0.75 g bis-acrylamide per 100 ml sterile water) and 3 ml of 0.3 M phosphate buffer (i.e. 0.15 M sodium dihydrogen phosphate, 0.15 M disodium hydrogen phosphate). After degassing the mixture, 25 µl TEMED catalyst and 250 µl 10% ammonium persulfate were added. The mixture was then poured into plastic tubes and the top of each gel was covered with a meniscus of water.

To prepare 7.5% gels, the following were gently mixed together: 11.7 ml sterile water, 15 ml of "7.5% PAG stock solution" (15 g acrylamide plus 0.375 g bis-acrylamide per 100 ml sterile water) and 3 ml of 0.3 M phosphate buffer. Further treatment and other additions were as above.

The dimensions of the gels were 0.6 cm (diameter) x 9 cm (length). The gels were allowed to set for 30 minutes and then were pre-run for 1 hour with a current of 7 milliamperes (mA) per gel.

The RNA samples to be electrophorezed were dissolved in water. An equal volume of solution D (1.2 g RNase-free sucrose + 2 ml 0.3 M phosphate buffer + 8 ml water) was added. The samples were then layered onto the gel surfaces using glass micropipettes. Electrophoresis was carried out in 0.03 M phosphate buffer for 80 minutes with a current of 7 mA per gel.

The gels were scanned for optical density at 260 mµ using a Joyce-Loebl (England) Scan 400 gel scanner with a Joyce-Loebl D7

power supply and a Bryans Southern Instruments (England) 28000 chart recorder.

CHAPTER 3

PREPARATION OF THE ENZYME FRACTION

As mentioned in Section 1.5, the major objectives of this study were to investigate and characterize an enzyme activity present in "uninfected" eukaryotic cells which promoted the incorporation of ^{3}H-UMP into TCA-precipitable material. Section 3.1 deals with the preliminary fractionation of the RNA-dependent activity. Section 3.2 deals with phosphocellulose chromatography of the crude enzyme fraction.

3.1 Preliminary fractionation

3.1.1 Introduction

3.1.1.1 Choice of system

Liver from specific pathogen-free (SPF) male Wistar rats was selected for study. The reasons are as follows: (a) male Wistar rats were readily available; (b) the stock population (bred at the John Curtin School of Medical Research) was relatively homogeneous from a genetic standpoint (syngeneic) and therefore only minimal variations in the results due to genetic differences between individuals would be expected; (c) specific pathogen-free animals would minimize the possibility of contamination by viral or bacterial enzymes; (d) the size of the liver in the animals made hepatectomy a relatively easy operation; and finally, (e) a few groups (Wilkie and Smellie, 1968a,b; Naora, 1975) reported an RNA-dependent enzyme activity in a crude fraction from rat liver which incorporated ^{3}H-UMP into a

trichloroacetic acid-(TCA-) precipitable product.

3.1.1.2 Choice of preparation method

The groups mentioned above both indicated that the activity may represent the synthesis of RNA heteropolymers as well as homopolymers. Wilkie and Smellie (1968b) merely suggested that the possibility of some formation of some heteropolymer could not be eliminated. Naora (1975), on the other hand, reported hybridization between the product synthesized by his enzyme fraction and the nuclear RNA added to the assay system. Although not demonstrating eukaryotic RNA replication, this latter report clearly suggested that such a reaction could take place in "uninfected" eukaryotes. The procedure for enzyme preparation used by Naora (1975) was derived from that used by Haruna et al. (1972) in preparing RNA-dependent RNA polymerase from leukemia and ascites tumour cells. This procedure was used as a starting point for the establishment of a method for preparing a crude enzyme fraction.

3.1.1.3 Evaluation of the preparation method

It was necessary to check, first, that the procedure would yield an activity which promoted the incorporation of ^{3}H-UMP into a TCA-precipitable product. The method would then be re-examined with a view to eliminating unnecessary steps and improving the yield since it was important, for the characterization of the enzyme activity, to firmly establish the procedure which would result in the best preliminary fractionation.

The solubilization of the enzyme activity with EDTA would be examined. An EDTA treatment was included in the preparation method of Haruna et al. (1972). The value of such a treatment would be assessed in preparing the enzyme fraction.

Ammonium sulfate precipitation is an efficient conventional technique in the preliminary fractionation of enzymes. The optimum ammonium sulfate "cut" for the preparation of the RNA-dependent enzyme activity had to be determined.

Finally, the RNA-dependence of the enzyme activity in the crude fraction had to be verified. A demonstration of the RNA-dependence of the reaction was essential to ensure that the method of preparation did yield an RNA-dependent activity. Once the crude fraction had been shown to meet this criterion, further investigations could be conducted.

3.1.2 Materials and methods

3.1.2.1 Standard preparation of the crude enzyme fraction

This procedure was described in Section 2.10 and outlined in Figure 2.1.

3.1.2.2 Definition of a unit of enzyme activity

A unit of enzyme activity was defined as that amount of protein which incorporated one picomole of UMP in 20 minutes at 36°C under the standard conditions of enzyme assay described in Section 2.12.

3.1.2.3 Calculation of enzyme specific activity

The specific activity of the enzyme preparations was obtained by dividing the number of units of enzyme activity present (see Section 3.1.2.2) by the number of milligrams of protein present (see Section 2.8).

3.1.2.4 EDTA treatment

One tenth volume of 0.11 M ethylenediamine tetraacetic acid (EDTA, disodium salt, Ajax Chemicals, Sydney), pH 7.4, was added to the supernatant obtained by centrifuging the homogenate (see Figure 2.1) at 17300 x g for 30 minutes. This was then stirred on ice for 5 minutes, and again centrifuged at 17300 x g for 30 minutes in the SS-34 rotor at 4°C. The supernatant of this second centrifugation was made 50% saturated with ammonium sulfate and the standard procedure was followed for the remainder of the preparation.

3.1.2.5 Ammonium sulfate fractionation

The supernatant of the 17300 x g centrifugation was saturated to 30% with solid ammonium sulfate, according to Green and Hughes (1955) (corrected for 0°C). Precipitation was allowed to take place with slow stirring on ice for 30 minutes. The solution was centrifuged at 3000 x g for 10 minutes. The pellet was placed on ice, while solid ammonium sulfate was added to the supernatant to bring it to 50% saturation. Precipitation was allowed to take place as before and the centrifugation was repeated. The "50% ammonium sulfate" pellet was also placed on ice, while the supernatant was brought to 80% saturation with solid ammonium sulfate. Precipitation

and centrifugation were as before. The "80% ammonium sulfate" pellet was also placed on ice, while the supernatant was completely saturated with ammonium sulfate. Precipitation and centrifugation were as indicated above. When the final pellet was collected, all precipitates were dissolved in Buffer B (see Section 2.10). They were then dialyzed overnight against Buffer B and assayed for enzyme activity as described in the text (Section 3.1.3.3).

3.1.3 Results

3.1.3.1 Yield of enzyme activity in the standard preparation

The yield of enzyme activity with the standard method of preparation (see Section 2.10) was measured. Aliquots of the homogenate, of the supernatant of the 17300 x g centrifugation and of the crude fraction were assayed for enzyme activity. Assays were performed in the standard assay mixture defined in Section 2.12, to which 14 µg nRNA and 34-44 µg of "enzyme" protein were added. ATP, GTP and CTP (0.4 mM each) and ammonium chloride (80 mM) were also added to the assay mixture. Incubation was for 20 minutes at 36°C. The protein content at each step of the preparation was determined and the total activity, specific activity, purification and % yield were calculated. The results are shown in Table 3.1.

The specific activity of the enzyme preparation increased from 50 in the homogenate to 130 in the crude enzyme fraction. The purification was therefore some 2.6-fold and the yield of enzyme was approximately 27% relative to the homogenate. The results demonstrated a small but definite increase in the purity of the enzyme.

Table 3.1 Standard preparation of enzyme

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Fold purification	Yield (%)
Homogenate	5,746	285,600	49.7	-	100
17300 x g supernatant	2,124	160,000	75.3	1.5	56
Crude fraction	592	76,212	128.7	2.6	26.7

3.1.3.2 EDTA treatment

Crude enzyme fractions were prepared from rat liver, both with and without an EDTA treatment. For each preparation, aliquots of the homogenate and of the crude fraction were assayed for enzyme activity, as in Section 3.1.3.1. The effect of the EDTA treatment on the yield of enzyme activity in the crude fraction is shown in Table 3.2, where the enzyme activity in the crude fraction is expressed as a percentage of the units of activity present in the homogenate.

Table 3.2 Effect of EDTA on the yield of enzyme activity[1]

Experiment No.	EDTA treatment	Enzyme activity in the crude fraction (% of units in the homogenate)	Average yield (%)
1	-	13	
2	-	13	14.3
3	-	17	
4	+	12	
5	+	16	14.0
6	+	14	

1. For assay conditions, see text (Section 3.1.3.1).

Since the EDTA treatment had no significant effect on the yield of enzyme activity in the crude fraction, it was not adopted.

3.1.3.3 Ammonium sulfate fractionation

The supernatant of the 17300 x g centrifugation was fractionated by saturating it to, consecutively, 30%, 50%, 80% and 100% with ammonium sulfate. The fractions were collected and dialyzed against Buffer B, as per Section 3.1.2.5. They were then assayed for enzyme activity under standard assay conditions (Section 2.12) except that 28 µg nRNA and approximately 74 µg of protein from each fraction were added to the assay mixtures. ATP, GTP and CTP (0.4 mM each) and ammonium chloride (80 mM) were also present in the assay. Incubation was for 20 minutes at 36°C.

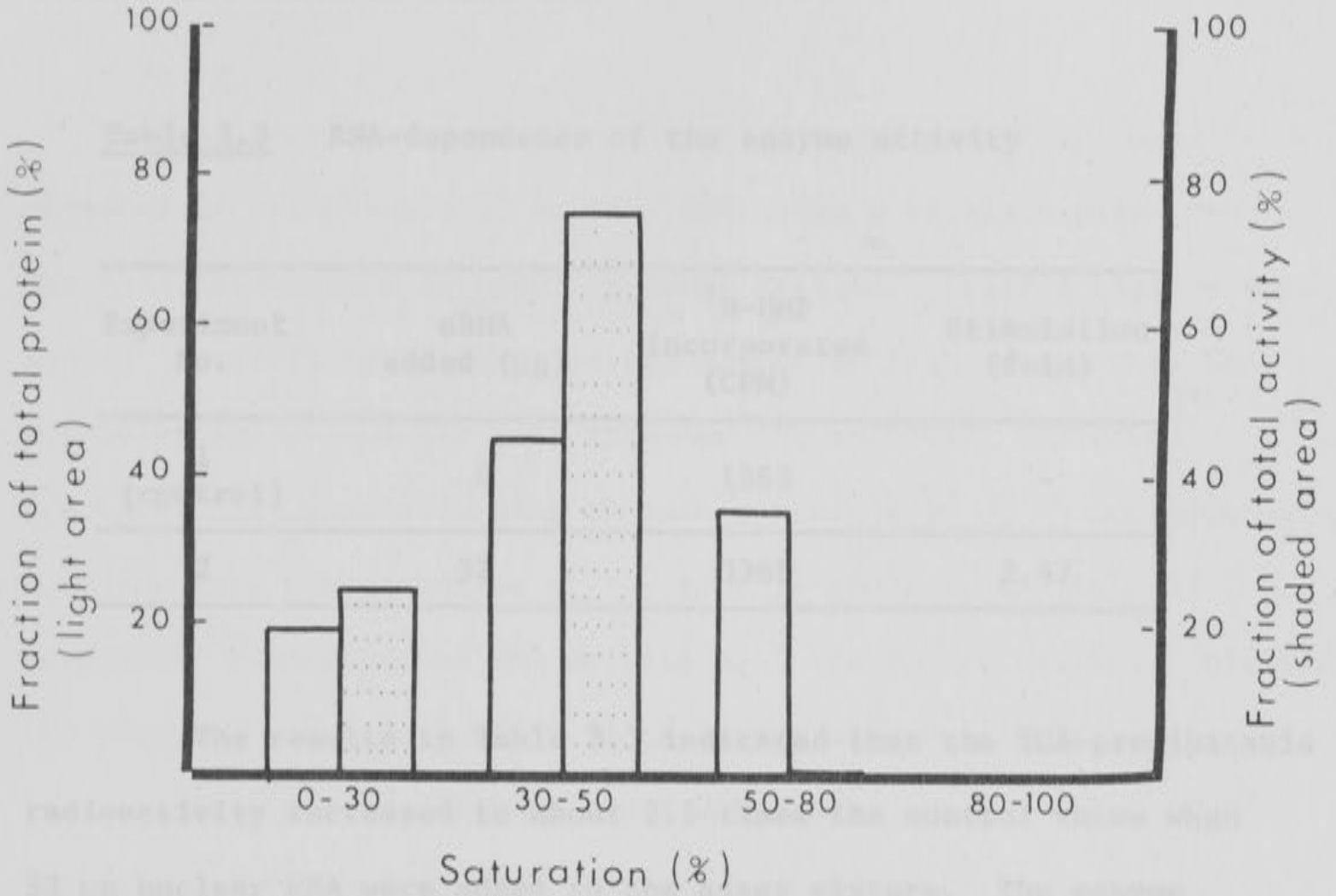
The relative amount of protein and the relative activity present in each fraction are shown in Figure 3.1. The amount of protein is expressed as a percentage of the total protein collected in the fractions and the enzyme activity is expressed as a percentage of the total units of activity present in the fractions.

As can be seen in Figure 3.1, almost all of the enzyme activity and only 65% of the total protein precipitated at 0-30% and 30-50% saturation with ammonium sulfate. The 50-80% ammonium sulfate "cut" resulted in the precipitation of a substantial amount of protein which had no significant enzyme activity. A single 0-50% ammonium sulfate"cut" was consequently adopted in the standard method of enzyme preparation.

3.1.3.4 RNA-dependence of the enzyme activity

The crude enzyme fraction was assayed for its ability to

Figure 3.1 Protein and enzyme activity obtained with various ammonium sulfate "cuts". Fractionation of protein and assay for enzyme activity were carried out as described in the text (Section 3.1.3.3).


100
80
60
40
20
Fraction of total protein (%)
(light area)
100
80
60
40
20
Fraction of total activity (%)
(shaded area)
0-30
30-50
50-80
80-100
Saturation (%)

promote in vitro the incorporation of ^{3}H-UMP into a TCA-precipitable product both in the presence and the absence of nuclear RNA. One hundred and fifty one µg of "crude fraction" protein plus 0 or 32 µg of nRNA were added to the assay mixture defined in Section 2.12. ATP, GTP and CTP (0.4 mM each) and ammonium chloride (80 mM) were also present in the assay. Incubation was for 20 minutes at 36°C. The results are shown in Table 3.3.

Table 3.3 RNA-dependence of the enzyme activity

Experiment No.	nRNA added (µg)	^{3}H-UMP incorporated (CPM)	Stimulation (fold)
1 (control)	0	1363	-
2	32	3365	2.47

The results in Table 3.3 indicated that the TCA-precipitable radioactivity increased to about 2.5 times the control value when 32 µg nuclear RNA were added to the assay mixture. The enzyme activity was not absolutely dependent upon the addition of exogenous RNA, as shown by the radioactivity obtained in the control. This may be due to the presence of endogenous RNA in the crude fraction.

The results obtained in this experiment suggested that an RNA-dependent enzyme activity was present in the crude fraction.

3.1.4 Discussion

The standard method of enzyme preparation was derived from

that of Haruna et al. (1972) and Naora (1975). With a slight modification, the method utilized by the above groups was suitable for the preparation from rat liver of an enzyme fraction which could promote in vitro the RNA-dependent incorporation of ^{3}H-UMP into a TCA-precipitable product. This modification consisted of omitting the EDTA treatment. The latter proved superfluous in preparing the crude enzyme fraction from rat liver.

A large amount of TCA-precipitable radioactivity could be obtained in the absence of nuclear RNA. One possible explanation for this observation is that the crude fraction itself contained RNA. Another possibility is that the endogenous activity was due to DNA-dependent RNA polymerase activity(-ies) contaminating the crude fraction. The standard assay mixture contained 10 µg/ml actinomycin D, since this concentration of the drug is known to inhibit the eukaryotic DNA-dependent RNA polymerase I (nucleolar enzyme). However, the presence of actinomycin D in the assay mixture did not guarantee that no DNA-dependent RNA synthesis would take place. Sobell (1974) recently ascertained that actinomycin D preferentially binds alternating G-C base sequences in DNA. No transcription from DNA can thus take place in the vicinity of a locus where alternating G-C sequences are bound to actinomycin. Transcription might take place, however, where this local requirement is not met.

The 2.5-fold increase in TCA-precipitable radioactivity observed when nuclear RNA was added to the assay mixture suggested that the crude fraction could catalyze a reaction which was RNA-dependent. A possibility which could not be excluded at this stage

was that the added nuclear RNA competed with endogenous RNA and/or enzyme products for ribonucleases present as contaminants in the crude fraction. This situation might also result in an increase in the TCA-precipitable radioactivity, since the endogenous incorporation of ^{3}H-UMP might thus be enhanced. This possibility is discussed further in Section 5.4.

3.2 Phosphocellulose chromatography

3.2.1 Introduction

3.2.1.1 The choice of phosphocellulose as the ion-exchanger

The RNA-dependent enzyme activity under study presumably utilized a ribonucleic acid chain as a template or as a primer. Therefore, this enzyme would be expected to possess some positively charged groups used in binding to RNA. This suggested the use of an anion-exchanger in the further fractionation of the enzyme activity.

As can be seen in Table 3.4, the use of phosphocellulose as anion-exchanger has proven successful in the purification of many nucleic acid polymerizing enzymes.

The chloride ion is often used as the counter-ion in phosphocellulose chromatography. The increase in potassium chloride concentration, which effects desorption of material from phosphocellulose, is brought about in one of two ways. Either a gradient of potassium chloride in buffer is applied to the column, or the potassium chloride concentration in the buffer is increased in a stepwise manner.

Table 3.4 Nucleic acid polymerizing enzymes which have been purified using phosphocellulose chromatography with potassium chloride-containing buffers.

A - Eukaryotic enzymes

	Enzyme	Source	Elution from P11 (M KCl)	Reference
1.	DNA-dependent DNA polymerase	Rabbit reticulocyte	0.25	Byrnes et al. (1974)
2.	DNA-dependent DNA polymerase "C"	Human acute lymphoblastic leukemia	0.28	McCaffrey et al. (1973)
3.	DNA-dependent DNA polymerase "I"	Normal human-blood lymphocytes	0.22	Smith and Gallo (1972)
4.	DNA-dependent DNA polymerase "N"	Human acute lymphoblastic leukemia	0.45	McCaffrey et al. (1973)
5.	DNA-dependent DNA polymerase "II"	Normal human-blood lymphocytes	0.45	Smith and Gallo (1972)
6.	DNA-dependent DNA polymerase β	Avian erythrocytes	0.35	Longacre and Rutter (1977)
7.	DNA-dependent DNA polymerase α	Avian erythrocytes	0.32	Longacre and Rutter (1977)
8.	DNA-dependent DNA polymerase (not previously reported)	Avian erythrocytes	0.25	Longacre and Rutter (1977)
9.	DNA polymerase A (R-DNA polymerase)	Human acute lymphoblastic leukemia	0.3	McCaffrey et al. (1973)
10.	Terminal deoxynucleotidyl transferase (DNA polymerase T)	Human acute lymphoblastic leukemia	0.33	McCaffrey et al. (1973)

Table 3.4 (continued)

	Enzyme	Source	Elution from P11 (M KCl)	Reference
A - Eukaryotic enzymes				
11.	RNA-dependent DNA polymerase	Human leukemic cells	I: 0.1 II: 0.28	Desai *et al.* (1974)
12.	RNA-dependent RNA polymerase	Rabbit reticulocytes	0.45	Downey *et al.* (1973)
13.	Poly(A) polymerase	Calf thymus	0.5	Winters and Edmonds (1973)
B - Prokaryotic enzymes				
14.	DNA-dependent RNA polymerase	*B. subtilis*	0.4	Clark *et al.* (1974)
15.	DNA-dependent RNA polymerase ("core polymerase")	*E. coli*	0.35	Burgess (1969a,b)
16.	σ factor	*E. coli*	0.05	Burgess *et al.* (1969)
17.	Adenosine triphosphate-ribonucleic acid adenyltransferase	*P. putida*	0.7	Payne and Boezi (1970)
C - Viral enzymes				
18.	DNA-dependent RNA polymerase	T7-infected *E. coli*	0.20 0.28 0.38	Niles *et al.* (1974)
19.	DNA-dependent RNA polymerase	PBS2-infected *B. subtilis*	0.55	Clark *et al.* (1974)
20.	RNA-directed DNA polymerase	AMV	0.3-0.4	Verma and Baltimore (1974)

Entry 11 in Table 3.4A refers to an enzyme activity in "transformed" cells. Since the agent responsible for the transformation has not been identified, however, the activity is provisionally included as a "eukaryotic enzyme".

3.2.1.2 Gradient elution: general remarks

Continuous gradients cause substances to be eluted from the column in symmetrical peaks and usually give better resolution. For these reasons, gradients are normally preferred to stepwise increases in salt concentration. In the case of multi-component enzyme systems, however, the application of a gradient may not prove entirely satisfactory. If some of the components of the enzyme system are only loosely bound to the phosphocellulose while others are tightly bound, the various components will elute at different salt concentrations. The enzyme activity will appear to have been lost until enzyme reconstitution experiments reveal which fractions of the eluate contain the various "components" or "subunits" required for enzyme activity. The very large number of fractions normally collected in phosphocellulose chromatography with an applied gradient makes such reconstitution experiments impractical.

3.2.1.3 Stepwise elution: general remarks

An alternative method of eluting material bound to phosphocellulose is by stepwise increases in the potassium chloride concentration of the buffer. The various fractions obtained with this method contain many different proteins and the purification of one protein species is consequently not as great as with gradient elution. However, stepwise elution is sometimes preferable where multi-component enzyme systems are being fractionated. One advantage which stepwise elution has over gradient elution is its simplicity, from a technical point of view.

3.2.1.4 Phosphocellulose chromatography in the preparation of the RNA-dependent activity

The possibility existed that the RNA-dependent enzyme activity observed in the crude fraction prepared from rat liver could be purified through phosphocellulose chromatography with gradient elution. This was suggested by the report of Downey et al. (1973) concerning the purification by this means of an RNA-dependent RNA polymerase from rabbit reticulocytes. On the other hand, Boyd and Fitschen (1975), working on an RNA-dependent enzyme activity from avian erythrocytes, reported that the method of Downey et al. (1973) was unsuccessful in their hands. Since the potential benefits were so great, the fractionation of the rat liver activity was attempted by means of phosphocellulose chromatography with a potassium chloride gradient of 0.05-1 M, as per Downey et al. (1973).

Alternatively, phosphocellulose chromatography was performed with stepwise elution of bound material.

3.2.2. Materials and methods

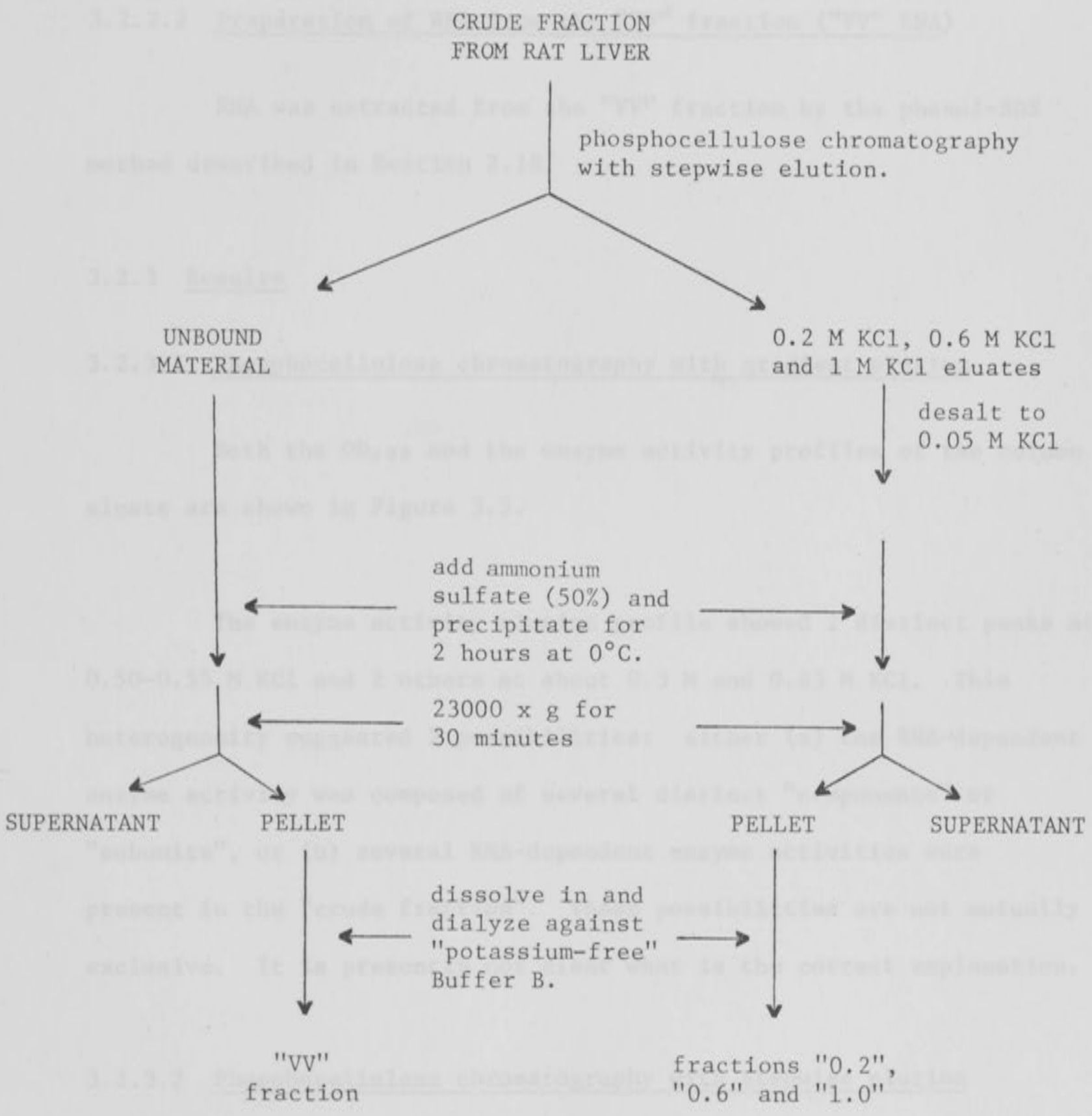
3.2.2.1 Phosphocellulose chromatography

The procedure for phosphocellulose chromatography with gradient elution was as follows: 56 mg of "crude fraction" protein (see Section 2.10) in 1 ml of Buffer B were applied to a 1.8 cm (diameter) x 9.5 cm (height) column of phosphocellulose, which was previously equilibrated with Buffer B. The unbound material was washed through with Buffer B and a potassium chloride gradient of 0.05-1.00 M in Buffer B (300 ml total volume) was then applied. The OD_{280} of the eluate was monitored

with an Isco (Lincoln, Nebraska) UA-5 absorbance monitor using a flow cell with a 10 mm light path. Two ml fractions were collected throughout with an Isco "Golden Retriever" fraction collector. Aliquots of 0.020 ml from several fractions were assayed for enzyme activity under the standard assay conditions (Section 2.12). Incubation was for 20 minutes at 36°C.

For chromatography with stepwise elution, "crude fraction" protein was loaded onto the column as described above. Bound material was then eluted with, alternately, 0.2 M, 0.6 M and 1.0 M KCl in Buffer B. The OD_{280} of the eluate was monitored as above. The material eluting from the column with 0.2 M KCl (volume of about 20 ml), that eluting with 0.6 M KCl (volume of about 35 ml) and that eluting with 1.0 M KCl (volume of about 25 ml) were desalted to 0.05 M KCl either by Sephadex (Pharmacia, Sweden) G-25 gel filtration or by overnight dialysis against 2 l Buffer B. The fraction containing unbound material and those containing the 0.2 M, 0.6 M and 1.0 M KCl eluates were made to 50% saturation with ammonium sulfate (Malinckrodt, St. Louis) which had been adjusted to pH 7.4 by the addition of ammonia solution (Ajax Chemicals, Melbourne). Precipitation was allowed to take place for 2 hours at 0°C. The precipitates were collected by centrifugation at 23000 x g for 30 minutes in the Sorvall RC2-B with the HB-4 rotor. The pellets were then dissolved in 0.2 ml of "potassium-free" Buffer B (0.05 M Tris-HCl, pH 7.8, 1 mM 2-mercapto-ethanol, 1 mM EDTA, 20% glycerol) and dialyzed for 3 hours against the same. The material thus recovered from the flow-through fraction is hereafter referred to as the "VV" ("void volume") fraction, while that recovered from the 0.2 M KCl, 0.6 M KCl and 1.0 M KCl eluates

Figure 3.2 Preparation of the "VV" fraction and of fractions "0.2", "0.6" and "1.0".


CRUDE FRACTION
FROM RAT LIVER
phosphocellulose chromatography
with stepwise elution.
UNBOUND
MATERIAL
0.2 M KCl, 0.6 M KCl
and 1 M KCl eluates
desalt to
0.05 M KCl
add ammonium
sulfate (50%) and
precipitate for
2 hours at 0°C.
23000 x g for
30 minutes
SUPERNATANT
PELLET
PELLET
SUPERNATANT
dissolve in and
dialyze against
"potassium-free"
Buffer B.
"VV"
fraction
fractions "0.2",
"0.6" and "1.0"

are referred to as fractions "0.2", "0.6" and "1.0", respectively. The preparation of these fractions is illustrated in Figure 3.2.

3.2.2.2 Preparation of RNA from the "VV" fraction ("VV" RNA)

RNA was extracted from the "VV" fraction by the phenol-SDS method described in Section 2.18.

3.2.3 Results

3.2.3.1 Phosphocellulose chromatography with gradient elution

Both the OD_{280} and the enzyme activity profiles of the column eluate are shown in Figure 3.3.

The enzyme activity elution profile showed 2 distinct peaks at 0.50-0.55 M KCl and 2 others at about 0.3 M and 0.85 M KCl. This heterogeneity suggested 2 possibilities: either (a) the RNA-dependent enzyme activity was composed of several distinct "components" or "subunits", or (b) several RNA-dependent enzyme activities were present in the "crude fraction". These possibilities are not mutually exclusive. It is presently not clear what is the correct explanation.

3.2.3.2 Phosphocellulose chromatography with stepwise elution

3.2.3.2.1 Multiple increases in potassium chloride concentration

This procedure was carried out as per Section 3.2.2.1 and a typical OD_{280} elution profile is shown in Figure 3.4. The fractions referred to as "VV", "0.2", "0.6" and "1.0" were assayed for enzyme

Figure 3.3 Phosphocellulose chromatography with gradient elution. Enzyme activity is expressed in terms of the ^{3}H-UMP incorporation (CPM) obtained with 0.020 ml of each fraction assayed. See text for method of assay.

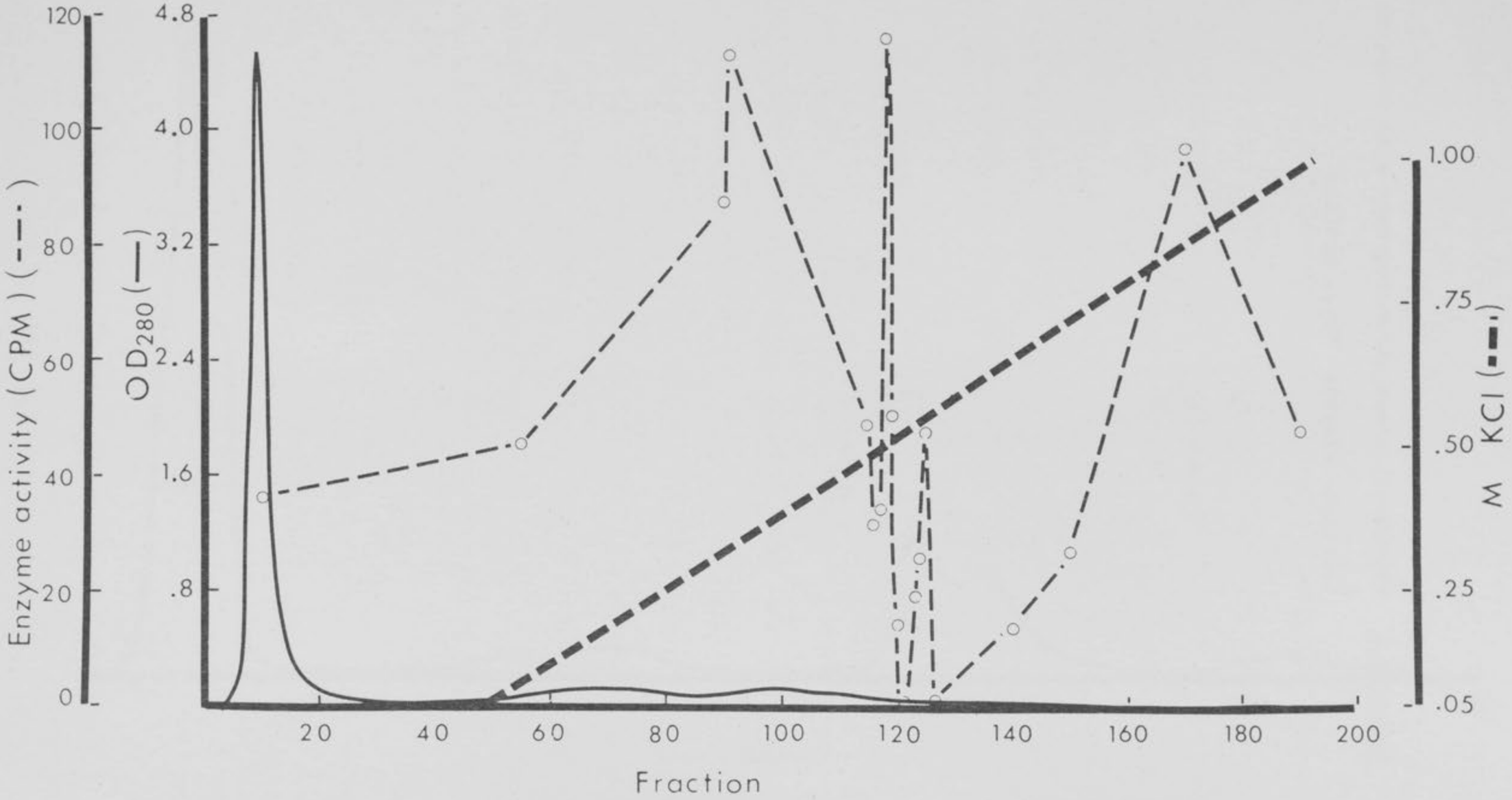
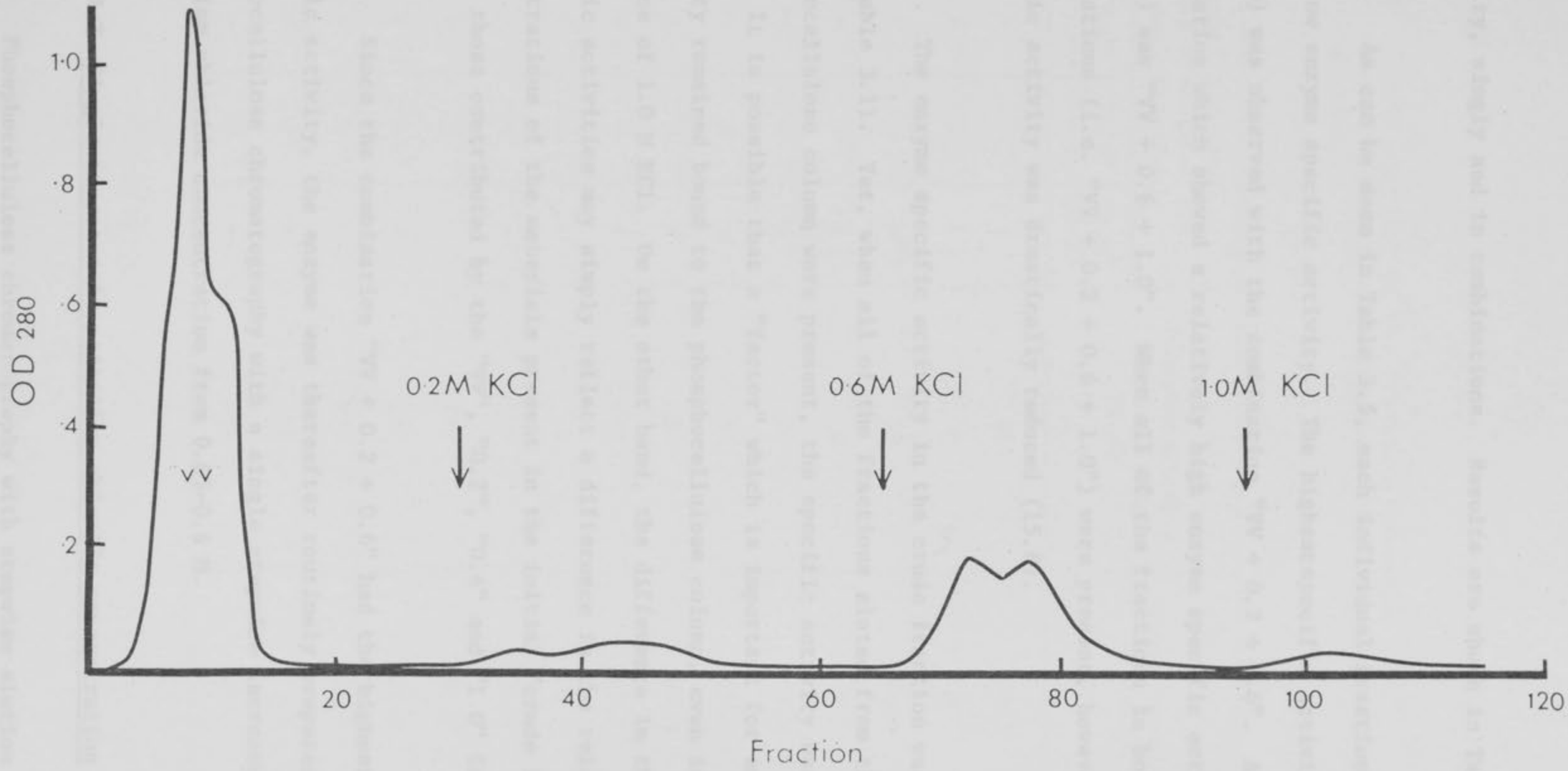

Enzyme activity (CPM) (- - -)
OD280 (——)
M KCl (- - -)
120
100
80
60
40
20
0
4.8
4.0
3.2
2.4
1.6
.8
1.00
.75
.50
.25
.05
20
40
60
80
100
120
140
160
180
200
Fraction

Figure 3.4 Phosphocellulose chromatography with stepwise elution (multiple steps): OD_{280} profile.


1·0
·8
·6
·4
·2
OD 280
VV
0·2M KCl
0·6M KCl
1·0M KCl
20
40
60
80
100
120
Fraction

activity, singly and in combinations. Results are shown in Table 3.5.

As can be seen in Table 3.5, each individual fraction had a very low enzyme specific activity. The highest specific activity (153.0) was observed with the combination "VV + 0.2 + 0.6". Another combination which showed a relatively high enzyme specific activity (121.4) was "VV + 0.6 + 1.0". When all of the fractions in both these combinations (i.e. "VV + 0.2 + 0.6 + 1.0") were present, however, the specific activity was drastically reduced (15.8).

The enzyme specific activity in the crude fraction was 128.7 (see Table 3.1). Yet, when all of the fractions eluted from the phosphocellulose column were present, the specific activity was only 15.8. It is possible that a "factor" which is important for enzyme activity remained bound to the phosphocellulose column, even in the presence of 1.0 M KCl. On the other hand, the difference in these specific activities may simply reflect a difference in the relative concentrations of the materials present in the initial "crude fraction" and of those contributed by the "VV", "0.2", "0.6" and "1.0" fractions.

Since the combination "VV + 0.2 + 0.6" had the highest enzyme specific activity, the enzyme was thereafter routinely prepared by phosphocellulose chromatography with a single stepwise increase in potassium chloride concentration from 0.05-0.6 M.

3.2.3.2.2 Single increase in potassium chloride concentration

Phosphocellulose chromatography with stepwise elution was carried out as per Section 3.2.2.1, except that bound material was

Table 3.5 Enzyme activity of the fractions recovered from phosphocellulose chromatography with stepwise elution.

"Enzyme" addition (μg protein from each fraction)				TCA-precipitable radioactivity (CPM)	Enzyme specific activity
"VV" (15.8)	"0.2" (5.9)	"0.6" (11.4)	"1.0" (3.8)		
+	-	-	-	61	16.5
-	+	-	-	0	0
-	-	+	-	49	18.6
-	-	-	+	0	0
+	+	-	-	29	5.8
+	-	+	-	340	53.9
+	-	-	+	384	84.3
-	+	+	-	473	117.7
-	+	-	+	0	0
-	-	+	+	102	28.9
+	+	+	-	1177	153.0
+	+	-	+	131	22.0
+	-	+	+	874	121.4
-	+	+	+	6	1.2
+	+	+	+	136	15.8

eluted with a single stepwise increase in KCl concentration from 0.05 M to 0.6 M. A typical OD_{280} profile is shown in Figure 3.5. The 0.6 M KCl eluate was treated as in Figure 3.2 and the resulting fraction is hereafter referred to as the "0.6 M" fraction. Theoretically, this "0.6 M" fraction was equal to the "0.2" fraction plus the "0.6" fraction of Section 3.2.3.2.1.

The enzyme specific activity and total enzyme activity in the fractions obtained from phosphocellulose chromatography with a single stepwise increase in potassium chloride concentration are shown in Table 3.6, as is the recovery of enzyme activity in these fractions. The latter is expressed as a percentage of the enzyme activity which was applied to the column.

The recovery of enzyme activity in the eluate from this phosphocellulose chromatography, before ammonium sulfate precipitation (see Figure 3.2),was 97%, of which 18% were in the "VV" fraction and 79% in the "0.6 M" fraction. Upon ammonium sulfate precipitation of the material contained in these fractions, the yield decreased reproducibly to 6% in the "VV" fraction and 16% in the "0.6 M" fraction. Thus, both before and after ammonium sulfate precipitation, the highest total enzyme activity was found in the "0.6 M" fraction.

The ammonium sulfate precipitation step resulted in a slight increase in the specific activity of both the "VV" fraction (from 24.8 to 44.3) and the "0.6 M" fraction (from 108.7 to 123.2). The highest enzyme specific activity was found in the "0.6 M" fraction. This was in agreement with the earlier results obtained from phosphocellulose

Figure 3.5 Phosphocellulose chromatography with stepwise elution (single step): OD_{280} profile.

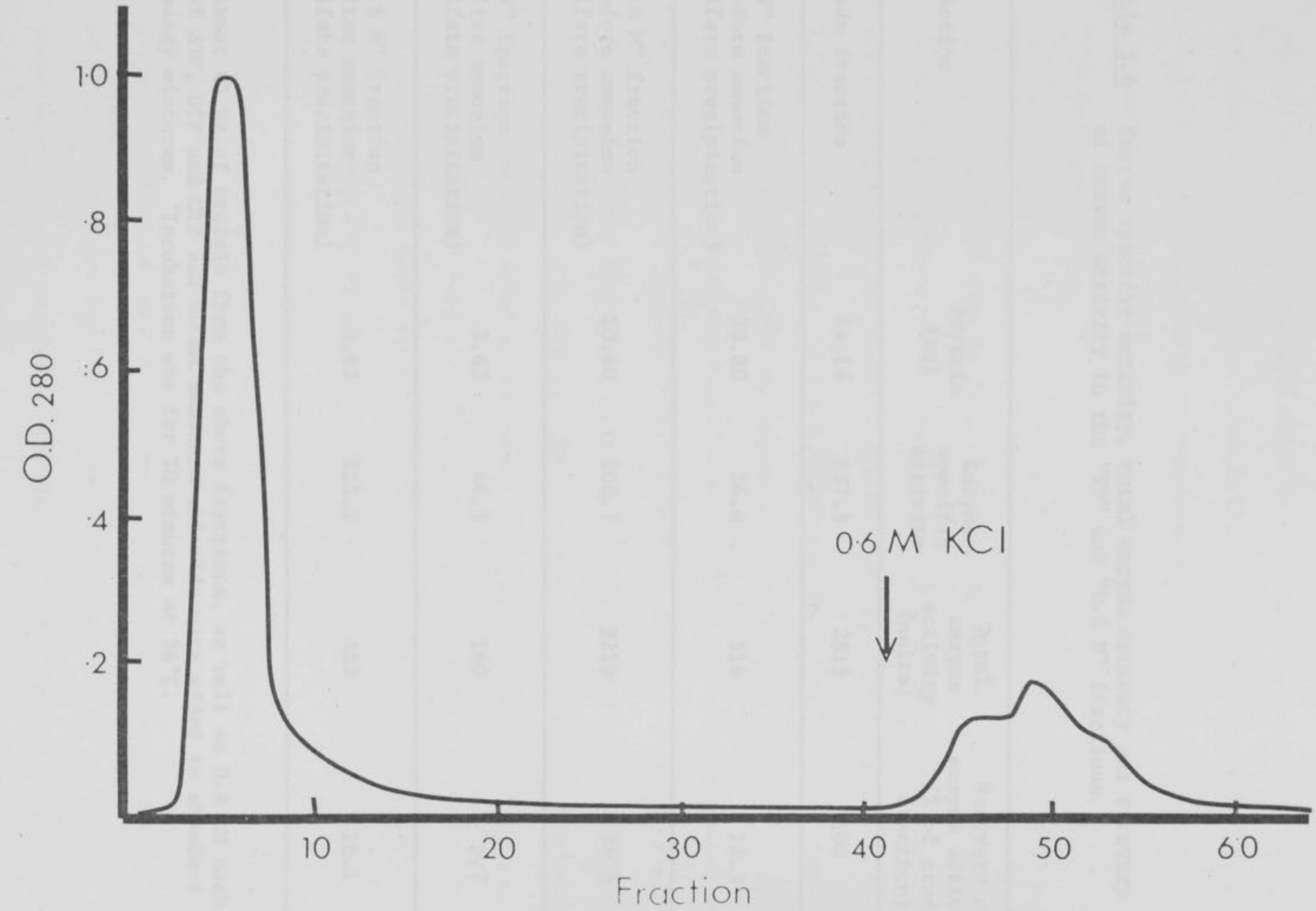

1·0
·8
·6
·4
·2
O.D. 280
0·6 M KCl
10
20
30
40
50
60
Fraction

Table 3.6 Enzyme specific activity, total enzyme activity and recovery of enzyme activity in the "VV" and "0.6 M" fractions.[1]

Fraction	Protein (mg)	Enzyme specific activity	Total enzyme activity (units)	Recovery of enzyme activity (% of crude fraction)
Crude fraction	44.16	127.5	2815	100
"VV" fraction (before ammonium sulfate precipitation)	20.80	24.8	516	18.3
"0.6 M" fraction (before ammonium sulfate precipitation)	20.40	108.7	2218	78.8
"VV" fraction (after ammonium sulfate precipitation)	3.62	44.3	160	5.7
"0.6 M" fraction (after ammonium sulfate precipitation)	3.67	123.2	452	16.1

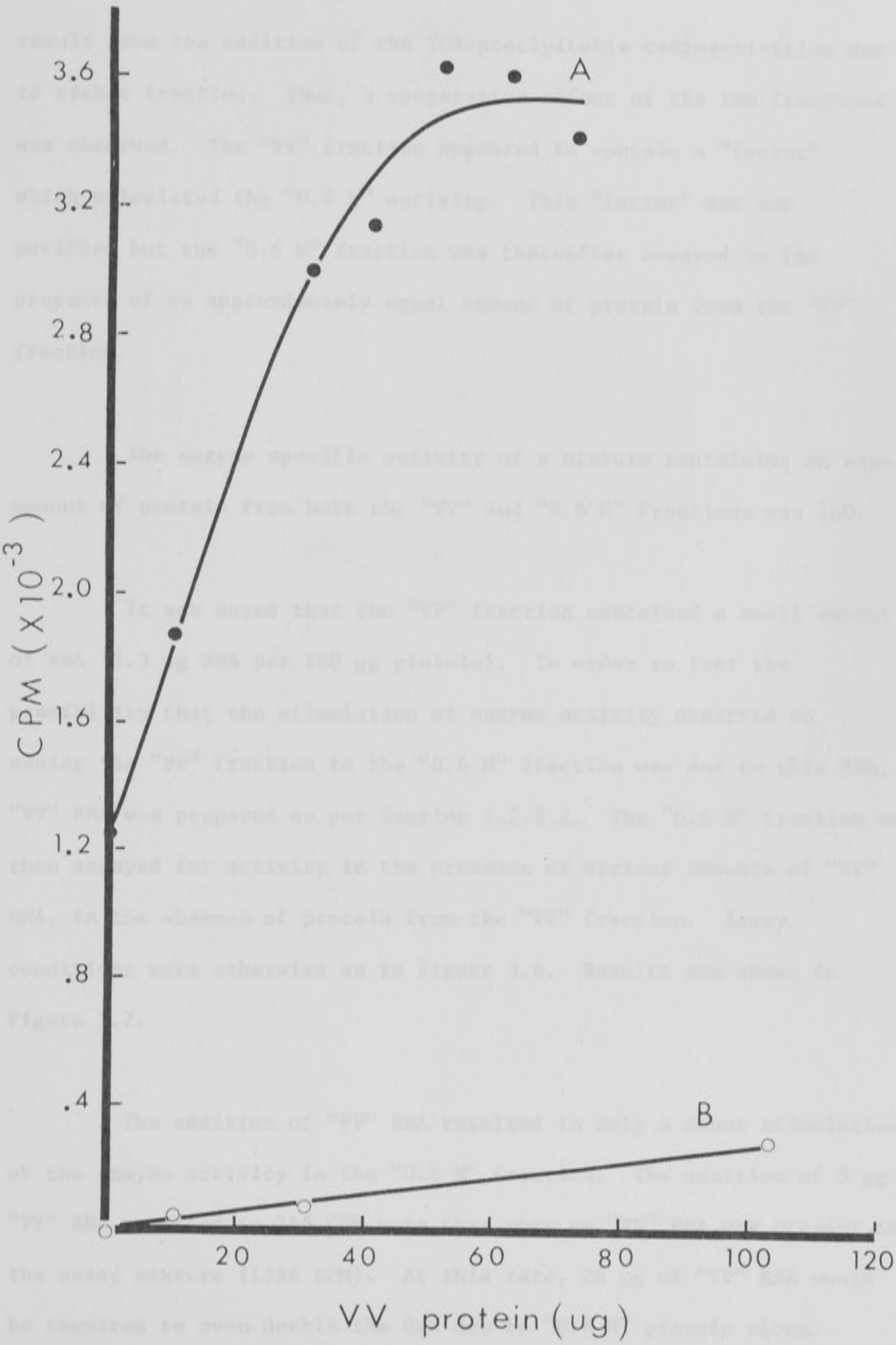
[1] About 45 µg of protein from the above fractions, as well as 0.4 mM each of ATP, GTP and CTP and 80 mM ammonium chloride were added to standard assay mixtures. Incubation was for 20 minutes at 36°C.

chromatography with multiple stepwise increases in the potassium chloride concentration (see Table 3.5). In that experiment, the "0.2 + 0.6" combination, which corresponds to the "0.6 M" fraction obtained here, also had a higher specific activity (117.7) than the "VV" fraction (16.5).

It can be seen in Table 3.5 that the "VV" fraction had a stimulatory effect on the activity of the "0.2 + 0.6" combination. Also, it increased the specific activity of the enzyme fraction (from 117.7 to 153.0). In order to determine if the "VV" fraction could similarly stimulate the enzyme activity present in the "0.6 M" fraction and increase the specific activity of the enzyme preparation, various amounts of protein from the "VV" fraction (0-73 µg) were added to standard assay mixtures containing a constant amount of "0.6 M" protein (45 µg). ATP, GTP and CTP (0.4 mM each) and ammonium chloride (80 mM) were also added to the assay mixture. As a control, varying amounts of protein from the "VV" fraction were added to mixtures identical to the above except that they contained no "0.6 M" protein. Incubation was for 20 minutes at 36°C. Results are shown in Figure 3.6.

The addition of increasing amounts of protein from the "VV" fraction to assay mixtures containing a constant amount of "0.6 M" protein resulted in increasing amounts of TCA-precipitable radioactivity. A plateau was reached when the amount of "VV" protein added approximately equalled the amount of "0.6 M" protein present in the assay mixture. At maximum stimulation, a 3-fold increase in enzyme activity was observed. The TCA-precipitable radioactivity obtained in the presence of both fractions was greater than would

Figure 3.6 Effect of the "VV" fraction on the "0.6 M" activity. Increasing amounts of protein from the "VV" fraction were added to assay mixtures containing either (A) 45 µg of "0.6 M" protein or (B) no "0.6 M" protein. See text for assay conditions.


3.6
3.2
2.8
2.4
2.0
1.6
1.2
.8
.4
CPM (X10-3)
A
B
20
40
60
80
100
120
VV protein (ug)

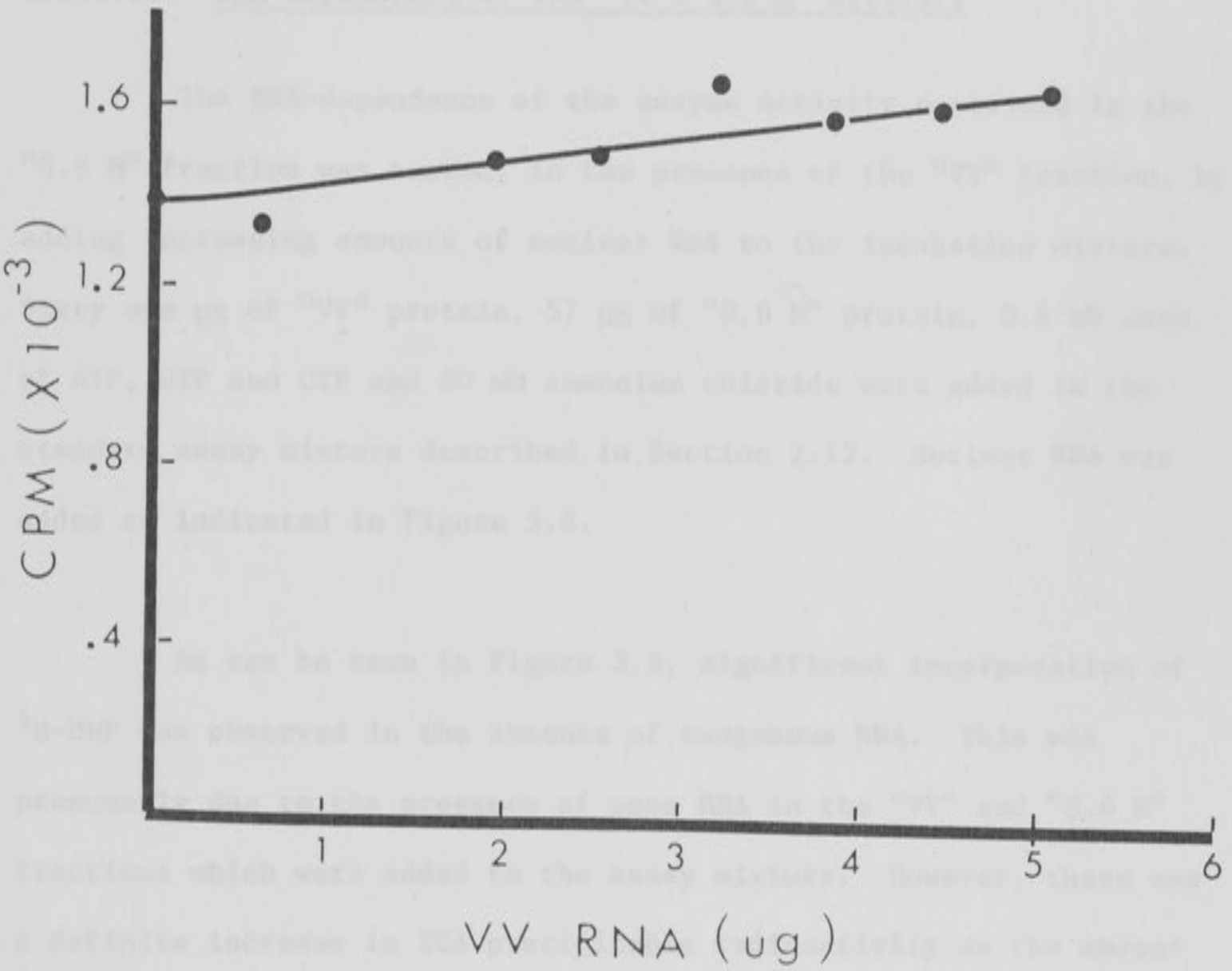
result from the addition of the TCA-precipitable radioactivities due to either fraction. Thus, a cooperative effect of the two fractions was observed. The "VV" fraction appeared to contain a "factor" which stimulated the "0.6 M" activity. This "factor" was not purified but the "0.6 M" fraction was thereafter assayed in the presence of an approximately equal amount of protein from the "VV" fraction.

The enzyme specific activity of a mixture containing an equal amount of protein from both the "VV" and "0.6 M" fractions was 160.

It was noted that the "VV" fraction contained a small amount of RNA (3.3 µg RNA per 100 µg protein). In order to test the possibility that the stimulation of enzyme activity observed on adding the "VV" fraction to the "0.6 M" fraction was due to this RNA, "VV" RNA was prepared as per Section 3.2.2.2. The "0.6 M" fraction was then assayed for activity in the presence of various amounts of "VV" RNA, in the absence of protein from the "VV" fraction. Assay conditions were otherwise as in Figure 3.6. Results are shown in Figure 3.7.

The addition of "VV" RNA resulted in only a minor stimulation of the enzyme activity in the "0.6 M" fraction. The addition of 5 µg "VV" RNA resulted in 265 CPM more than when no "VV" RNA was present in the assay mixture (1388 CPM). At this rate, 26 µg of "VV" RNA would be required to even double the CPM due to "0.6 M" protein alone. Since approximately 60 µg of protein from the "VV" fraction were normally added to the assay mixture, only about 2 µg of "VV" RNA were

Figure 3.7 Effect of "VV" RNA on the "0.6 M" activity. See text for assay conditions.


1.6
1.2
.8
.4
CPM (X 10-3)
1
2
3
4
5
6
VV RNA (ug)

present. This result clearly indicated that the "VV" RNA was not responsible for the observed stimulation of the "0.6 M" activity. This stimulation therefore had to be mediated by a component other than RNA, for example, a protein or lipoprotein.

3.2.3.2.3 RNA-dependence of the "VV + 0.6 M" activity

The RNA-dependence of the enzyme activity contained in the "0.6 M" fraction was tested, in the presence of the "VV" fraction, by adding increasing amounts of nuclear RNA to the incubation mixture. Sixty one µg of "VV" protein, 57 µg of "0.6 M" protein, 0.4 mM each of ATP, GTP and CTP and 80 mM ammonium chloride were added to the standard assay mixture described in Section 2.12. Nuclear RNA was added as indicated in Figure 3.8.

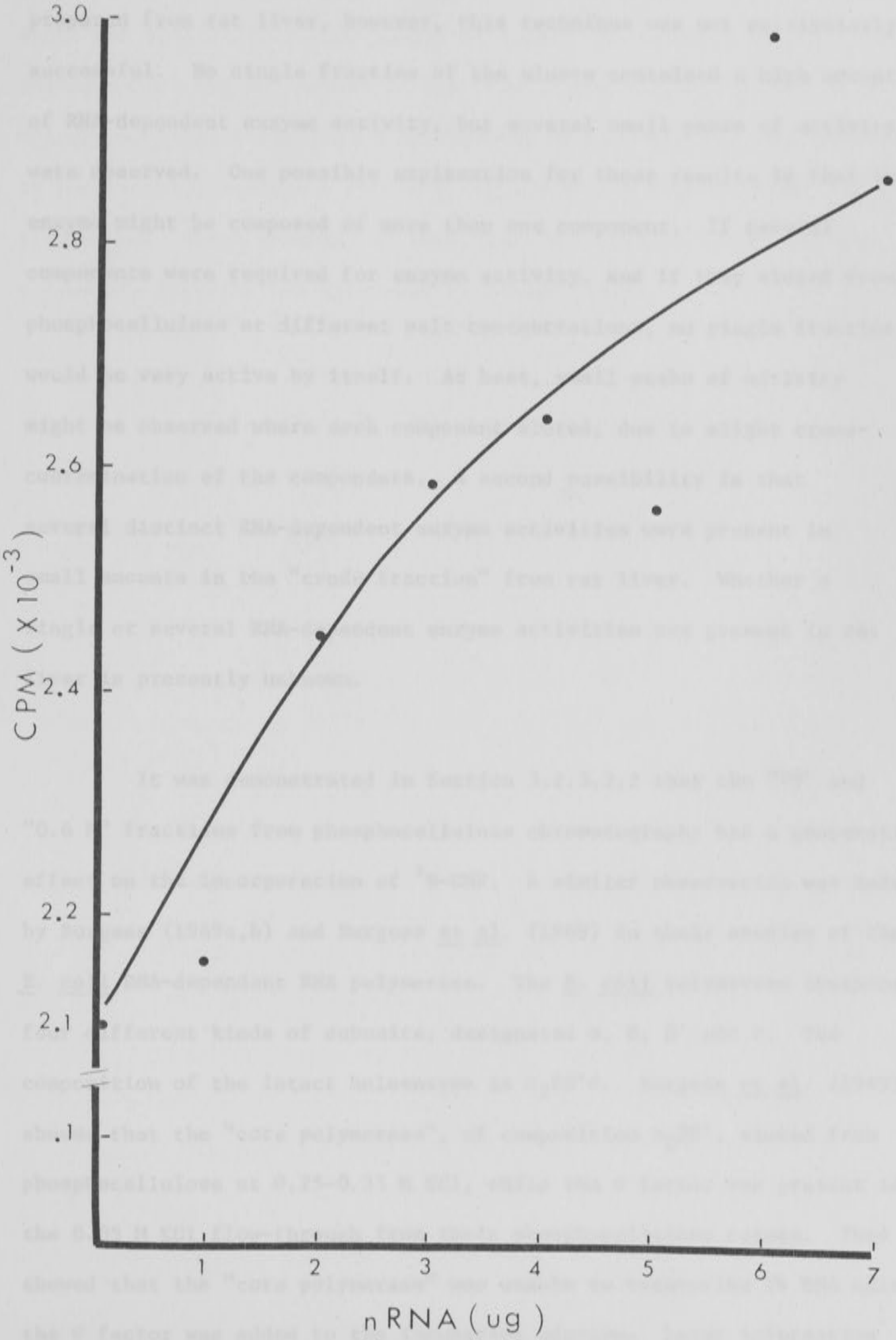
As can be seen in Figure 3.8, significant incorporation of ^{3}H-UMP was observed in the absence of exogenous RNA. This was presumably due to the presence of some RNA in the "VV" and "0.6 M" fractions which were added to the assay mixture. However, there was a definite increase in TCA-precipitable radioactivity as the amount of nuclear RNA which was added to the assay mixture was increased.

3.2.4 Discussion

The technique of phosphocellulose chromatography with gradient elution was used successfully by Downey et al. (1973) in the purification of a cytoplasmic, microsome-bound RNA-dependent RNA polymerase from rabbit reticulocyte lysates. The enzyme eluted from phosphocellulose at 0.45 M KCl. When applied to the "crude fraction"

Figure 3.8 RNA-dependence of the "VV + 0.6 M" activity.

See text for assay conditions.


3.0
2.8
2.6
2.4
2.2
2.1
.1
CPM (X 10^-3)
1
2
3
4
5
6
7
nRNA (ug)

prepared from rat liver, however, this technique was not particularly successful. No single fraction of the eluate contained a high amount of RNA-dependent enzyme activity, but several small peaks of activity were observed. One possible explanation for these results is that the enzyme might be composed of more than one component. If several components were required for enzyme activity, and if they eluted from phosphocellulose at different salt concentrations, no single fraction would be very active by itself. At best, small peaks of activity might be observed where each component eluted, due to slight cross-contamination of the components. A second possibility is that several distinct RNA-dependent enzyme activities were present in small amounts in the "crude fraction" from rat liver. Whether a single or several RNA-dependent enzyme activities are present in rat liver is presently unknown.

It was demonstrated in Section 3.2.3.2.2 that the "VV" and "0.6 M" fractions from phosphocellulose chromatography had a cooperative effect on the incorporation of ^{3}H-UMP. A similar observation was made by Burgess (1969a,b) and Burgess et al. (1969) in their studies of the E. coli DNA-dependent RNA polymerase. The E. coli polymerase contains four different kinds of subunits, designated α, β, β' and σ. The composition of the intact holoenzyme is $\alpha_2\beta\beta'\sigma$. Burgess et al. (1969) showed that the "core polymerase", of composition $\alpha_2\beta\beta'$, eluted from phosphocellulose at 0.25-0.35 M KCl, while the σ factor was present in the 0.05 M KCl flow-through from their phosphocellulose column. They showed that the "core polymerase" was unable to transcribe T4 DNA unless the σ factor was added to the incubation mixture. Later information revealed that the σ subunit was a regulatory protein. A protein factor

which can specifically bind to and stimulate the activity of rat liver DNA-dependent RNA polymerase II has been tentatively identified in the cytoplasm of rat liver (Stein and Hausen, 1970). Whether this protein is functionally analogous to the E. coli σ factor remains to be elucidated. Other examples of components which alter eukaryotic RNA polymerase activity are discussed by Roeder (1976).

CHAPTER 4

CHARACTERIZATION OF THE RNA-DEPENDENT ENZYME ACTIVITY

4.1 Introduction

It was shown in Chapter 3 that the enzyme fraction obtained from phosphocellulose chromatography could promote the RNA-dependent incorporation of ^{3}H-UMP into a TCA-precipitable product. The aim of this chapter is to characterize the enzyme activity. Among the characteristics investigated are the time course and pH optimum for UMP incorporation and the effects of monovalent and divalent cations. The effects of pronase, RNase A, DNase I, pyrophosphate and orthophosphate on the enzyme activity will be studied, as well as those of known inhibitors of RNA synthesis. The NTP preference and RNA specificity of the enzyme activity will also be examined.

4.2 Materials and methods

Sodium pyrophosphate was from Ajax Chemicals (Sydney). Sodium dihydrogen phosphate was from B.D.H. Chemicals Ltd. (England). Nuclease-free pronase was from Calbiochem (California). Aurintricarboxylic acid was from Eastman Kodak Co. (New York). Synthetic ribopolymers ("Takamine") were from Miles Chemical Company (Victoria). Alpha-amanitin, rifampicin and RNase A were from Sigma (St. Louis). DNase I was from Worthington (New Jersey).

4.2.1 Preparation of RNAs

The method of preparing the various RNAs was outlined in Chapter 2.

4.2.2 Method of enzyme assay

The method of enzyme assay was given in Section 2.12. Standard assay mixtures were used, except where otherwise indicated. In all assays, approximately equal amounts of protein (51-66 µg) from the "void volume" ("VV") and "0.6 M" fractions (Section 3.2.3.2.2) were used. This combination of "VV" and "0.6 M" fractions is hereafter referred to as the phosphocellulose enzyme.

4.3 Results

4.3.1 Time course

The time course of the reaction had to be ascertained, so that assays could be conducted for a period of time consonant with net incorporation of NMP. The time course of UMP incorporation was studied in the presence of both $MnCl_2$ and $MgCl_2$ and in the presence of either of these salts. Results are shown in Figure 4.1.

Net incorporation of ^{3}H-UMP into TCA-precipitable material took place during the first 90, 30 or 20 minutes of incubation in the presence of $MnCl_2$ plus $MgCl_2$, $MnCl_2$ or $MgCl_2$, respectively. Subsequent assays were conducted for 60 minutes in the presence of $MnCl_2$ plus $MgCl_2$ (except where otherwise indicated) and for 20 minutes in the presence of either $MnCl_2$ or $MgCl_2$.

Figure 4.1 Time course of UMP incorporation.

A. Presence of both $MnCl_2$ and $MgCl_2$.

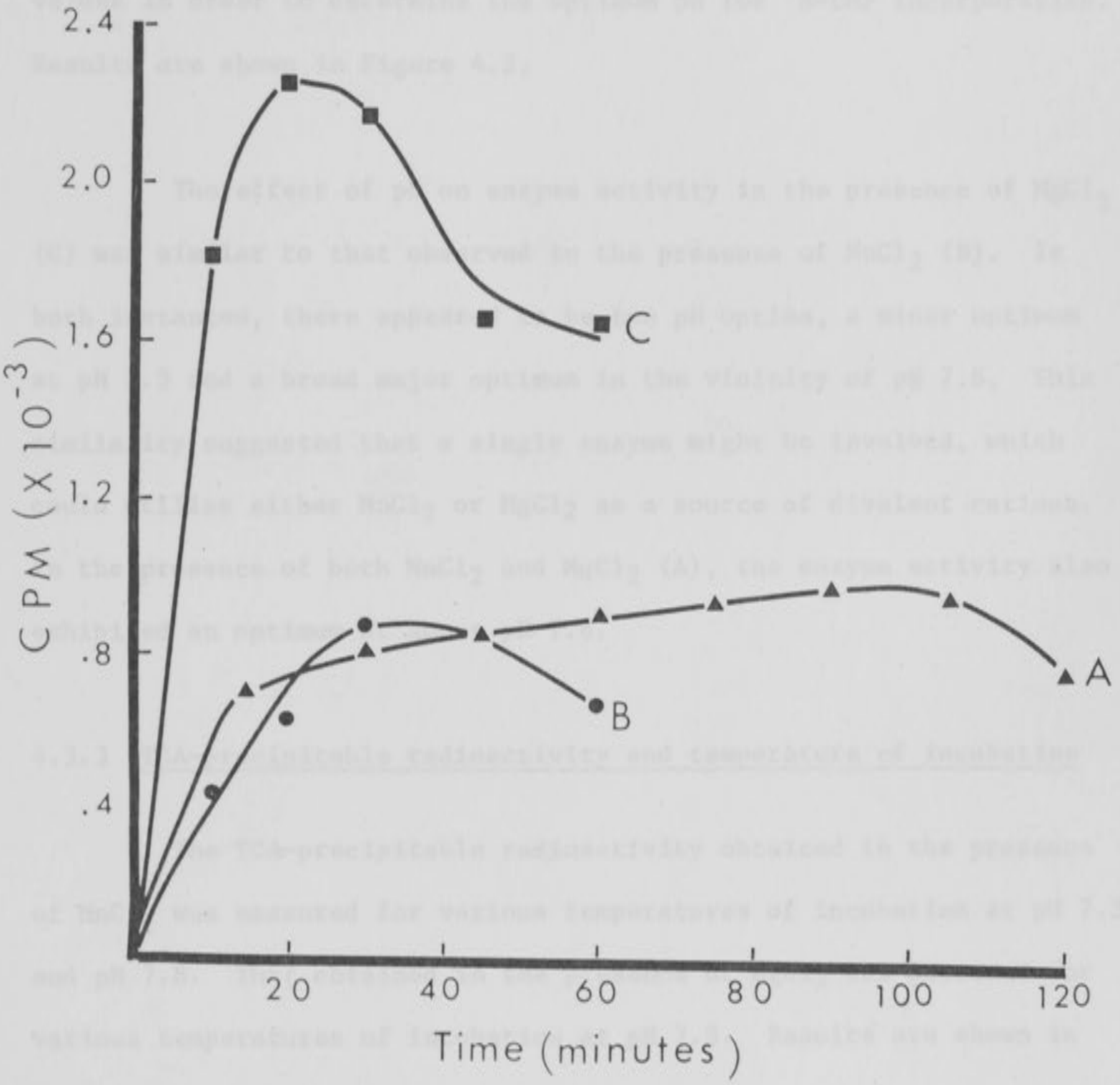
Eighty mM ammonium chloride plus 0.4 mM each of ATP, GTP and CTP were added to the standard assay mixture.

B. Presence of $MnCl_2$ only.

ATP, GTP and CTP (0.4 mM each) were added to the standard assay mixture. $MgCl_2$ was omitted.

C. Presence of $MgCl_2$ only.

$MnCl_2$ was omitted from the standard assay mixture.


2.4
2.0
1.6
1.2
.8
.4
CPM (X10-3)
C
A
B
20
40
60
80
100
120
Time (minutes)

4.3.2 pH optimum

The phosphocellulose enzyme was assayed at different pH values in order to determine the optimum pH for ^{3}H-UMP incorporation. Results are shown in Figure 4.2.

The effect of pH on enzyme activity in the presence of $MgCl_2$ (C) was similar to that observed in the presence of $MnCl_2$ (B). In both instances, there appeared to be two pH optima, a minor optimum at pH 7.3 and a broad major optimum in the vicinity of pH 7.8. This similarity suggested that a single enzyme might be involved, which could utilize either $MnCl_2$ or $MgCl_2$ as a source of divalent cations. In the presence of both $MnCl_2$ and $MgCl_2$ (A), the enzyme activity also exhibited an optimum at about pH 7.8.

4.3.3 TCA-precipitable radioactivity and temperature of incubation

The TCA-precipitable radioactivity obtained in the presence of $MnCl_2$ was measured for various temperatures of incubation at pH 7.3 and pH 7.8. That obtained in the presence of $MgCl_2$ was measured for various temperatures of incubation at pH 7.8. Results are shown in Figure 4.3.

At pH 7.8, maximum incorporation of ^{3}H-UMP took place at 30-35 °C when either $MnCl_2$ (A) or $MgCl_2$ (B) was present. These results again suggested that a single enzyme activity might be involved.

At pH 7.8, the Q_{10} of the enzyme activity in the presence of $MnCl_2$ was 1.9 (Figure 4.3A). A Q_{10} of about 2.0 is consistent with an

Figure 4.2 pH optimum

A. Presence of both $MnCl_2$ and $MgCl_2$.

ATP, GTP and CTP (0.4 mM each) were added to the standard assay mixture. The pH was as indicated in the abscissa.

B. Presence of $MnCl_2$ only.

$MgCl_2$ was omitted from the standard assay mixture.

C. Presence of $MgCl_2$ only.

$MnCl_2$ was omitted from the standard assay mixture.

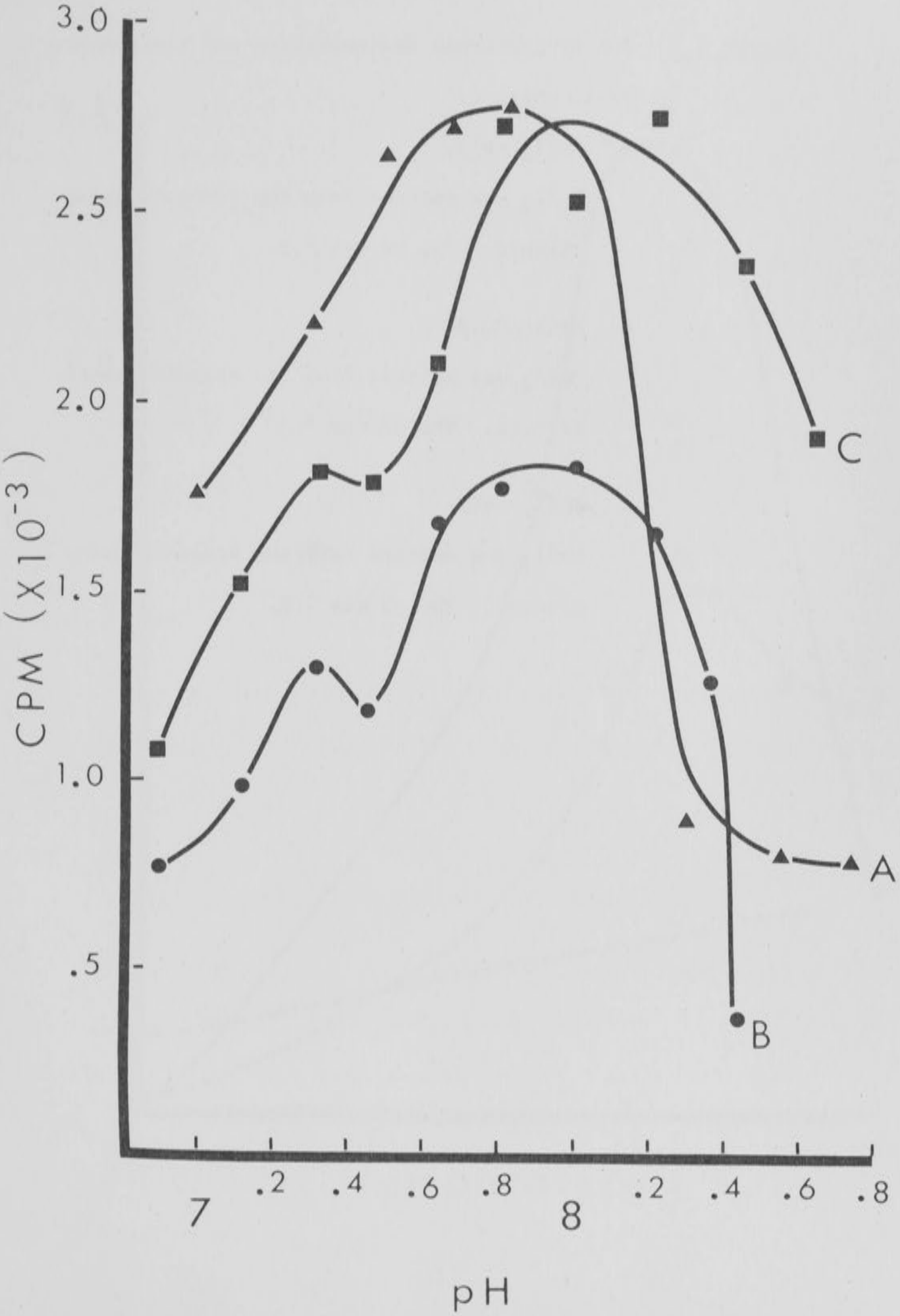

3.0
2.5
2.0
1.5
1.0
.5
CPM (X10-3)
A
B
C
7
.2
.4
.6
.8
8
.2
.4
.6
.8
pH

Figure 4.3 TCA-precipitable radioactivity and temperature of incubation.

A. $MnCl_2$ only.

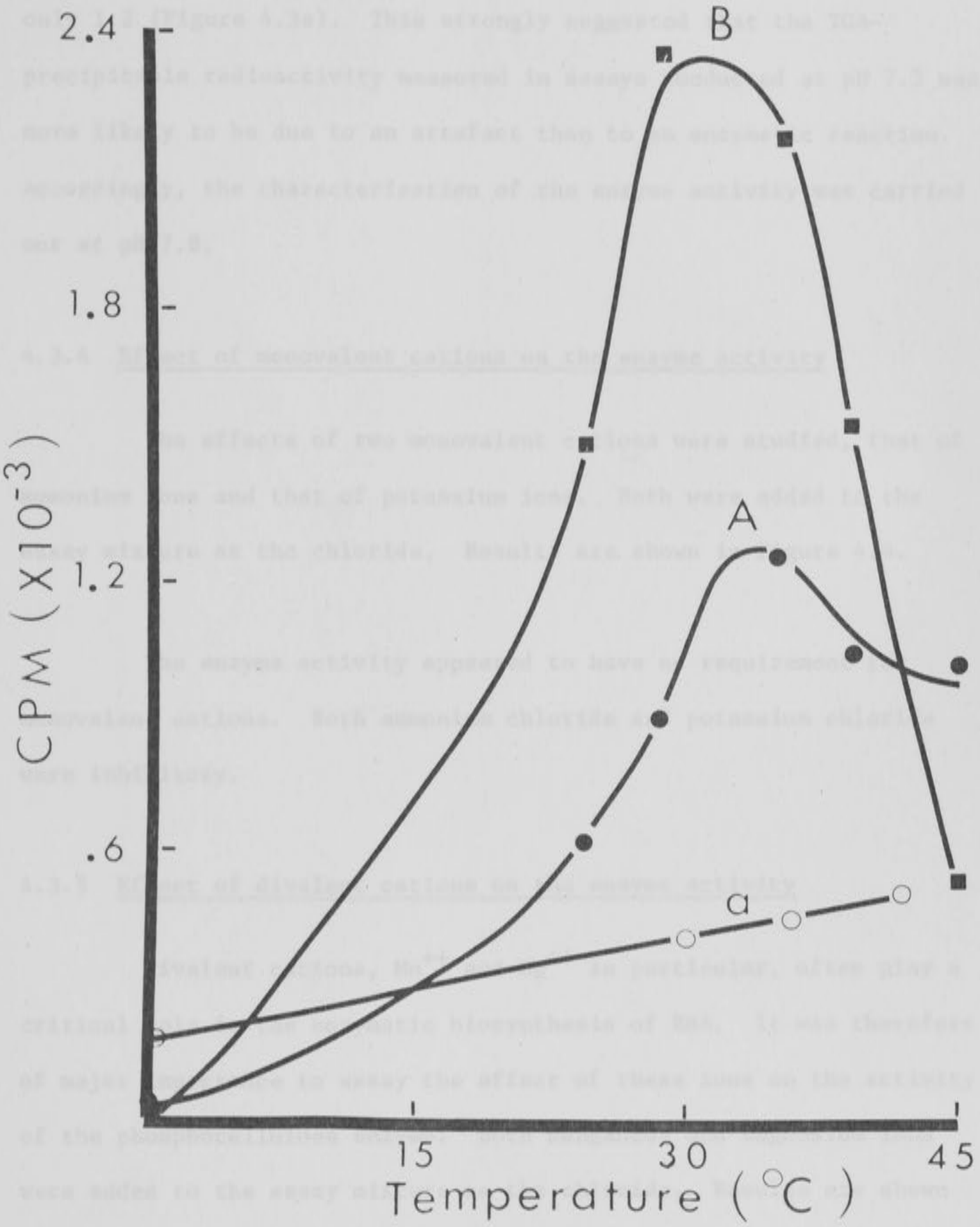
$MgCl_2$ was omitted from the standard assay mixture. The pH was 7.8.

a. $MnCl_2$ only.

$MgCl_2$ was omitted from the standard assay mixture. The pH was 7.3.

B. $MgCl_2$ only.

$MnCl_2$ was omitted from the standard assay mixture. The pH was 7.8.


2.4
1.8
1.2
.6
CPM (X10-3)
B
A
a
15
30
45
Temperature (°C)

enzymatic reaction (Lehninger, 1975). At pH 7.3, however, the Q_{10} was only 1.2 (Figure 4.3a). This strongly suggested that the TCA-precipitable radioactivity measured in assays conducted at pH 7.3 was more likely to be due to an artefact than to an enzymatic reaction. Accordingly, the characterization of the enzyme activity was carried out at pH 7.8.

4.3.4 Effect of monovalent cations on the enzyme activity

The effects of two monovalent cations were studied, that of ammonium ions and that of potassium ions. Both were added to the assay mixture as the chloride. Results are shown in Figure 4.4.

The enzyme activity appeared to have no requirement for monovalent cations. Both ammonium chloride and potassium chloride were inhibitory.

4.3.5 Effect of divalent cations on the enzyme activity

Divalent cations, Mn^{++} and Mg^{++} in particular, often play a critical role in the enzymatic biosynthesis of RNA. It was therefore of major importance to assay the effect of these ions on the activity of the phosphocellulose enzyme. Both manganese and magnesium ions were added to the assay mixture as the chloride. Results are shown in Figure 4.5.

Two $MnCl_2$ concentrations (Figure 4.5A) appeared to be optimal for ^{3}H-UMP incorporation, whether $MgCl_2$ was present (b) or absent (a). These were 1.3 mM and 3.9 mM $MnCl_2$.

Figure 4.4 Effect of monovalent cations.

Ammonium chloride (A) or potassium chloride (B) was added to standard assay mixtures, which also contained 0.4 mM each of ATP, GTP and CTP.

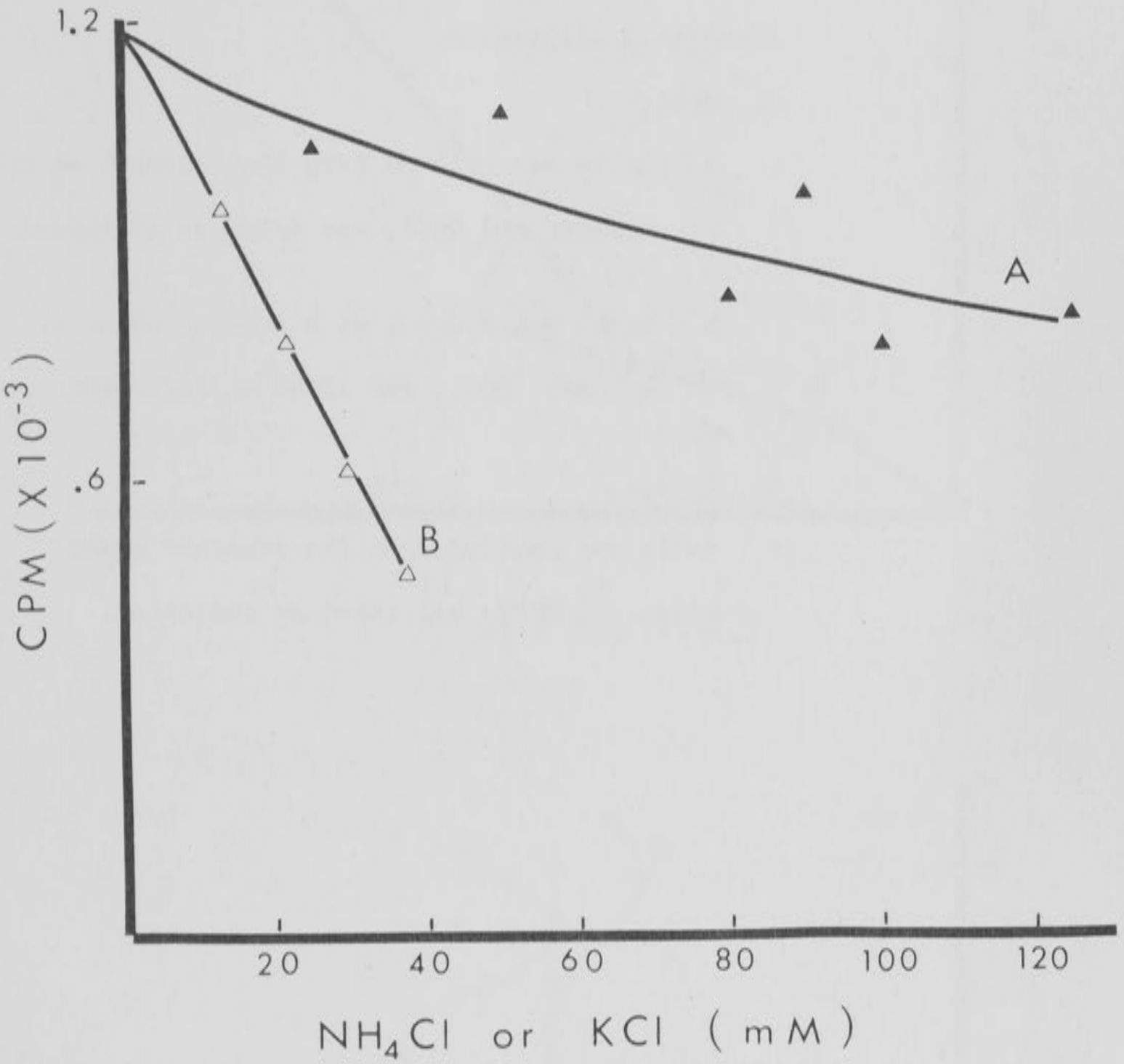

1.2
.6
CPM (X 10⁻³)
A
B
20
40
60
80
100
120
NH₄Cl or KCl (mM)

Figure 4.5 Effect of divalent cations.

ATP, GTP and CTP (0.4 mM each) were added to the standard assay mixture. Incubation was for 20 minutes in all assays.

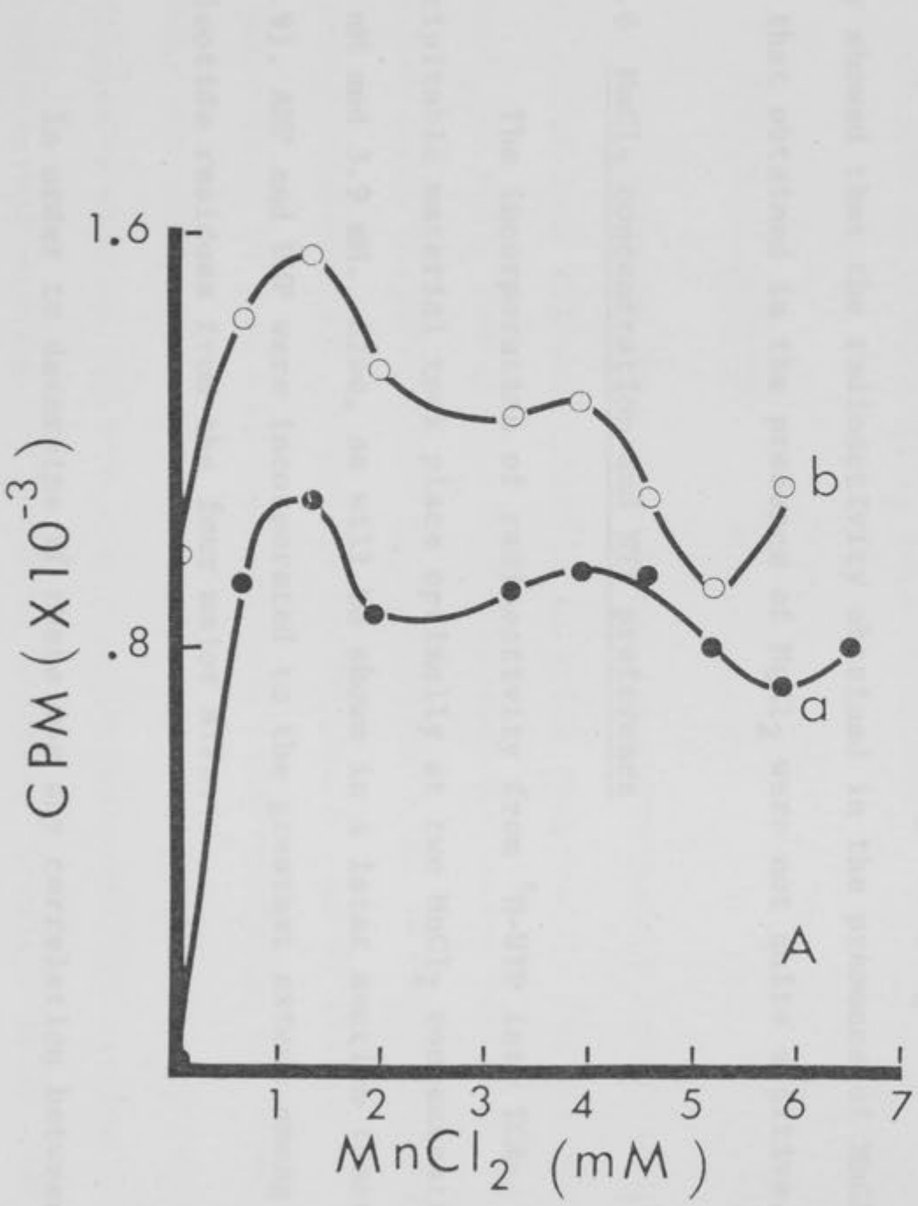
A. $MnCl_2$.

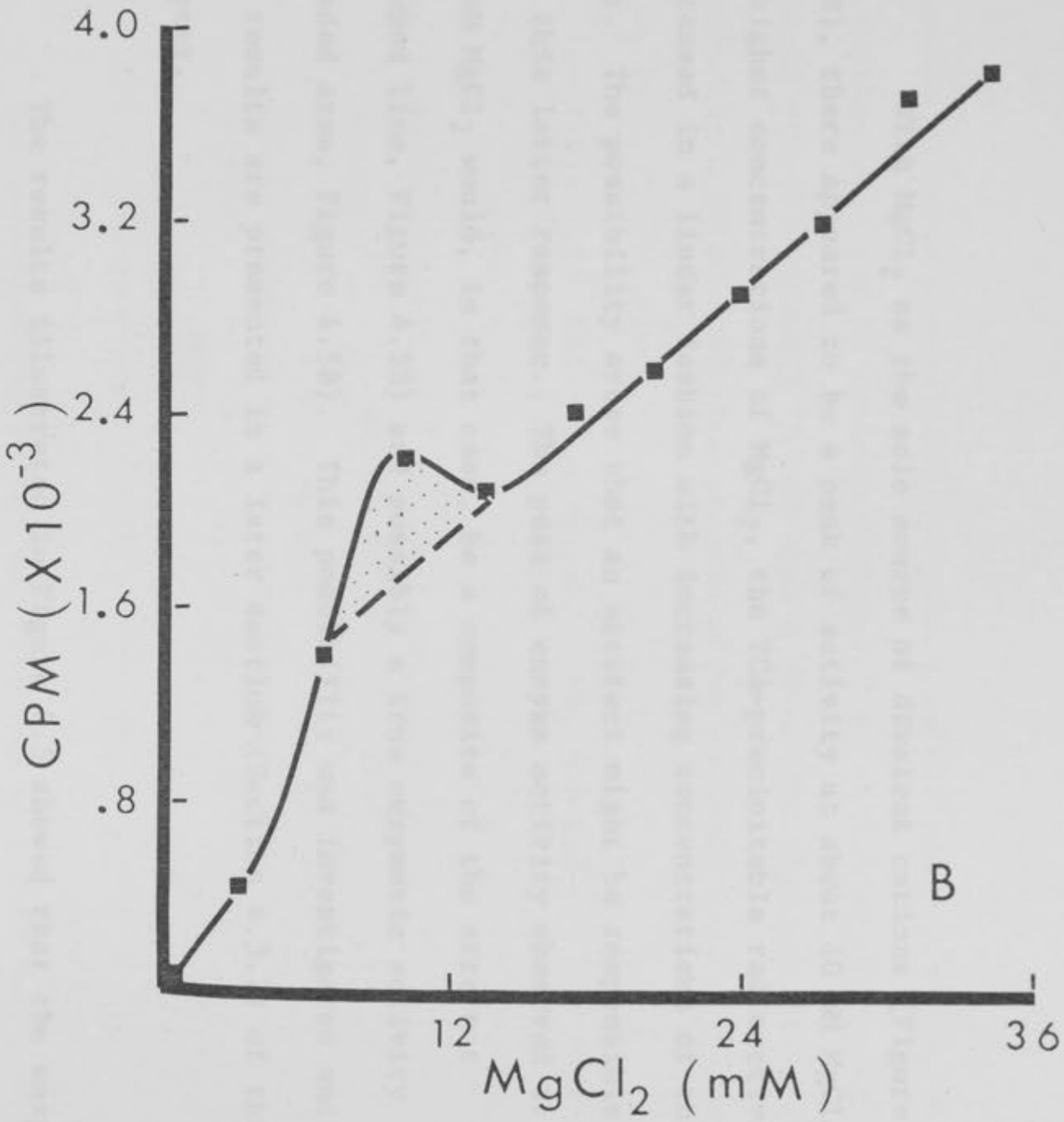
a. $MgCl_2$ was omitted from the standard assay mixture and $MnCl_2$ was added as indicated.

b. $MgCl_2$ was present at a concentration of 4.5 mM. $MnCl_2$ was added as indicated.

B. $MgCl_2$.

$MnCl_2$ was omitted from the standard assay mixture. $MgCl_2$ was added as indicated.


1.6
.8
CPM (X10^{-3})
b
a
A
1
2
3
4
5
6
7
MnCl$_2$ (mM)


4.0
3.2
2.4
1.6
.8
CPM (X10^{-3})
B
12
24
36
MgCl$_2$ (mM)

With $MgCl_2$ as the sole source of divalent cations (Figure 4.5B), there appeared to be a peak of activity at about 10 mM $MgCl_2$. At higher concentrations of $MgCl_2$, the TCA-precipitable radioactivity increased in a linear fashion with increasing concentrations of this salt. The possibility arose that an artefact might be responsible for this latter response. The peak of enzyme activity observed at 10 mM $MgCl_2$ would, in that case, be a composite of the artefact (dashed line, Figure 4.5B) and possibly a true enzymatic activity (shaded area, Figure 4.5B). This possibility was investigated and the results are presented in a later section (Section 4.3.7) of this chapter.

The results illustrated in Figure 4.5 showed that the enzyme activity had an absolute requirement for a divalent cation and that this requirement could be met with either $MnCl_2$ or $MgCl_2$. In addition, they showed that the radioactivity obtained in the presence of $MnCl_2$ and that obtained in the presence of $MgCl_2$ were not quite additive.

4.3.6 $MnCl_2$ concentration and NTP preference

The incorporation of radioactivity from ^{3}H-UTP into TCA-precipitable material took place optimally at two $MnCl_2$ concentrations: 1.3 mM and 3.9 mM. Also, as will be shown in a later section (Section 4.3.9), AMP and UMP were incorporated to the greatest extent among the nucleotide residues from the four major NTPs.

In order to determine if there was any correlation between $MnCl_2$ concentration and NTP preference, an assay was performed to measure the incorporation of AMP and UMP at 1.3 mM and 3.9 mM $MnCl_2$.

Magnesium chloride was omitted from the standard assay mixture, which was further modified to contain either 1.3 mM or 3.9 mM $MnCl_2$ and either 5 µCi (45 Ci/mmol) ^{3}H-UTP plus 0.08 mM UTP or 2 µCi (24 Ci/mmol) ^{3}H-ATP plus 0.08 mM ATP. Results are shown in Table 4.1.

Table 4.1 $MnCl_2$ concentration vs. AMP and UMP incorporation

Labelled substrate	pmoles NMP incorporated	
	1.3 mM $MnCl_2$	3.9 mM $MnCl_2$
^{3}H-ATP	23.04	16.86
^{3}H-UTP	2.80	5.99

The incorporation of AMP was favoured at 1.3 mM $MnCl_2$ and that of UMP was favoured at 3.9 mM $MnCl_2$, although there was more AMP than UMP incorporated at both $MnCl_2$ concentrations.

4.3.7 Effect of pronase

In order to determine if the incorporation of radioactivity from ^{3}H-UTP into TCA-precipitable material observed in the presence of $MnCl_2$ or $MgCl_2$ was enzyme-(and therefore protein-) mediated, standard assay mixtures minus $MgCl_2$ (Figure 4.6A) or $MnCl_2$ (Figure 4.6B) were pre-incubated for 12 minutes at 36°C with 1 mg/ml (final concentration) of nuclease-free pronase. Alternatively, nuclease-free pronase was added some time after the start of the incubation period, to determine whether the TCA-precipitable radioactivity was due to the artefactual binding of ^{3}H-UTP to protein. Possible mechanisms envisaged for such an artefact included "magnesium bridging" of ^{3}H-UTP

to protein and the physical trapping of ^{3}H-UTP within TCA-precipitated protein aggregates. In these experiments, ^{3}H-UTP was added at "zero-time" (Figure 4.6), whether the assay mixture had been pre-incubated with pronase or not. Results are shown in Figure 4.6, where vertical arrows denote the times of pronase addition.

A 12 minute pre-incubation with pronase completely eliminated TCA-precipitable radioactivity (line a in Figure 4.6A and B). This clearly demonstrated that the incorporation of ^{3}H-UMP into TCA-precipitable material was due to a protein. The temperature response shown in Section 4.3.3 suggested that this protein was an enzyme.

The addition of pronase some time after the start of the incubation period (line b in Figure 4.6A and B) did not reduce the amount of TCA-precipitable radioactivity which had been obtained at the time the addition was made. This showed that the TCA-precipitable radioactivity was not simply due to a protein-mediated artefact. Consequently, if the response of the enzyme activity to increasing amounts of $MgCl_2$ (Figure 4.5B) was artefactual, the artefact was not due to the presence of protein.

The addition of pronase after 30 minutes incubation, in the presence of $MnCl_2$ (line b, Figure 4.6A), and the addition of pronase after 20 minutes incubation, in the presence of $MgCl_2$ (line b, Figure 4.6B), resulted in a greater amount of TCA-precipitable radioactivity than in the corresponding controls (last point on the solid line, Figure 4.6A and B). This was possibly due to the digestion by pronase of the ribonuclease(s) which were probably endogenous to the

Figure 4.6 Effect of pronase.

A. Presence of $MnCl_2$ only.

$MgCl_2$ was omitted from the standard assay mixture.

Solid line: control (no pronase added).

Line a : pronase added 12 minutes before the addition of ^{3}H-UTP.

Line b : pronase added after 30 minutes incubation; mixture incubated for a further 30 minutes.

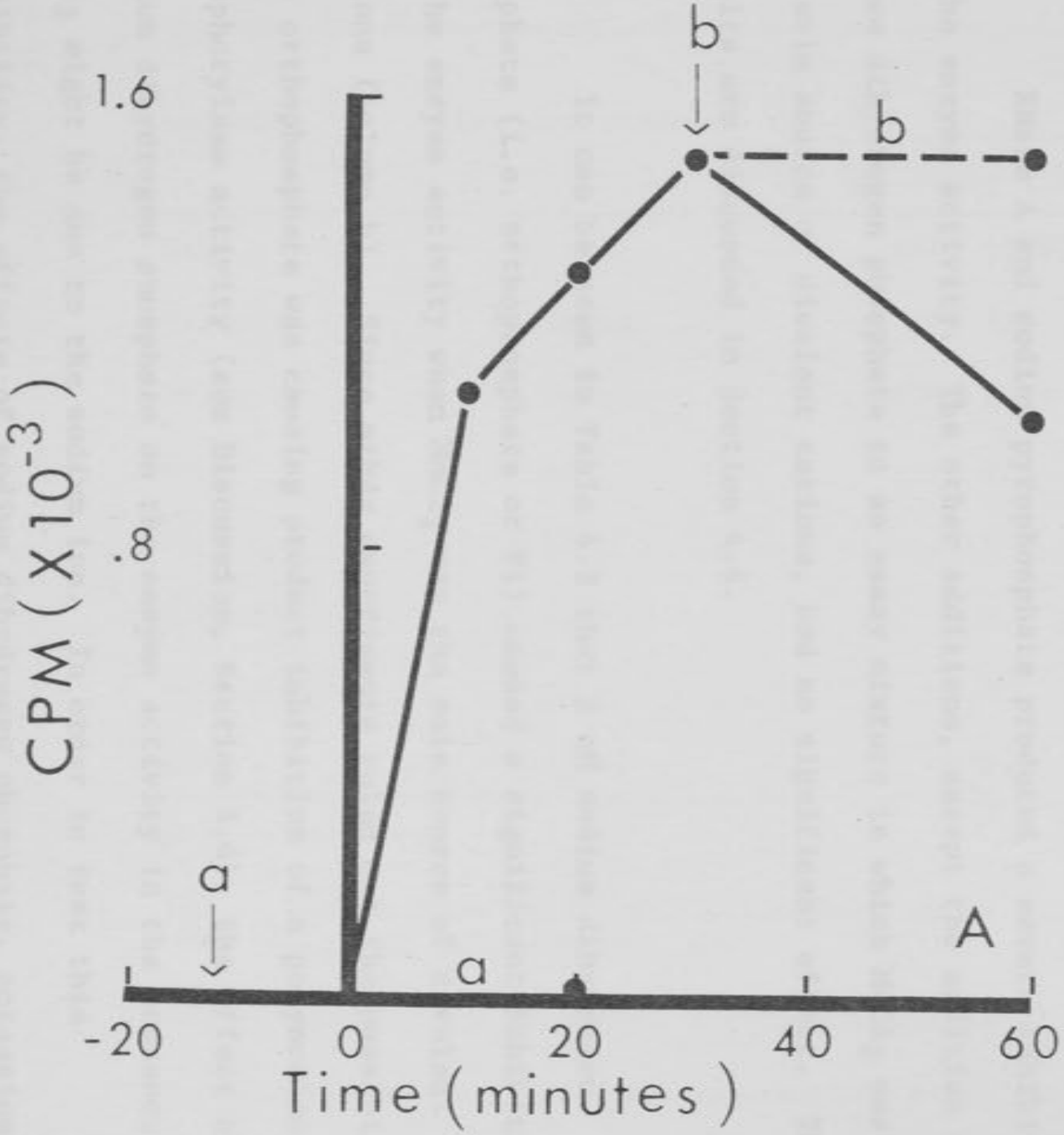
B. Presence of $MgCl_2$ only.

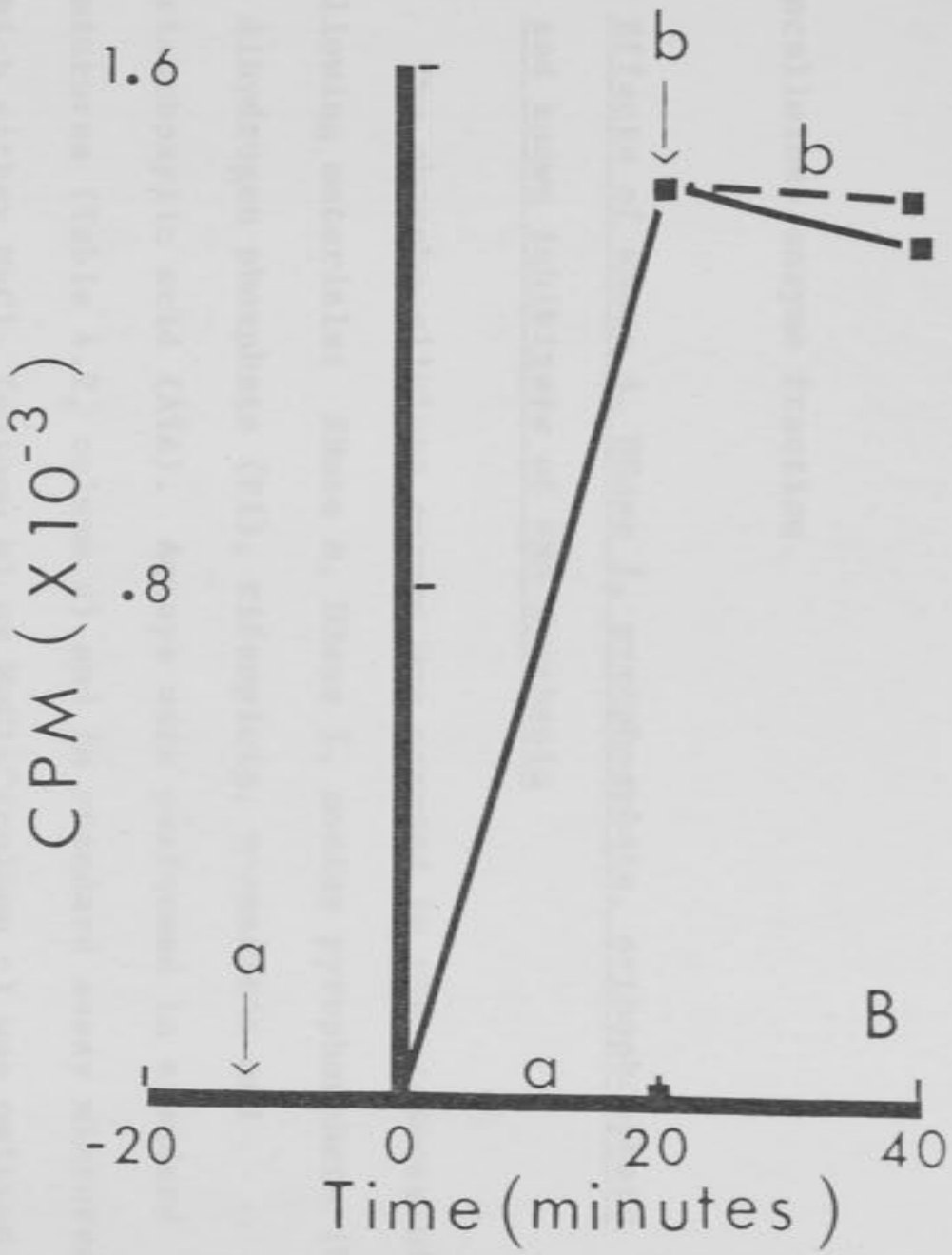
$MnCl_2$ was omitted from the standard assay mixture.

Solid line: control (no pronase added).

Line a : pronase added 12 minutes before the addition of ^{3}H-UTP.

Line b : pronase added after 20 minutes incubation; mixture incubated for a further 20 minutes.


1.6
CPM (X10-3)
.8
b
b
a
a
A
-20
0
20
40
60
Time (minutes)


1.6
CPM (X10-3)
.8
b
b
a
a
B
-20
0
20
40
Time (minutes)

phosphocellulose enzyme fraction.

4.3.8 Effects of RNase A, DNase I, pyrophosphate, orthophosphate and known inhibitors of RNA synthesis

The phosphocellulose enzyme was assayed in the presence of the following materials: RNase A, DNase I, sodium pyrophosphate (PPi), sodium dihydrogen phosphate (Pi), rifampicin, α-amanitin and aurintricarboxylic acid (ATA). Assays were performed in standard assay mixtures (Table 4.2, column a) and in standard assay mixtures from which either $MgCl_2$ (column b) or $MnCl_2$ (column c) was omitted. Results are shown in Table 4.2.

RNase A and sodium pyrophosphate produced a severe inhibition of the enzyme activity. The other additions, except the addition of sodium dihydrogen phosphate to an assay mixture in which $MnCl_2$ was the sole source of divalent cations, had no significant effect. These results are discussed in Section 4.4.

It can be seen in Table 4.2 that 2 mM sodium dihydrogen phosphate (i.e. orthophosphate or Pi) caused a significant inhibition of the enzyme activity when $MnCl_2$ was the sole source of divalent cations (column b). Since other experiments ruled out the possibility that orthophosphate was causing product inhibition of a polynucleotide phosphorylase activity (see Discussion, Section 4.4), the effect of sodium dihydrogen phosphate on the enzyme activity in the presence of $MnCl_2$ might be due to the sodium ion. In order to test this possibility, the effects of sodium dihydrogen phosphate, potassium dihydrogen phosphate and potassium chloride, iodide and bicarbonate

Table 4.2 Effects of RNase A, DNase I, pyrophosphate, orthophosphate and known inhibitors of RNA synthesis.

Addition	Concentration	TCA-precipitable radioactivity (CPM)		
		(a) $MnCl_2$ + $MgCl_2$	(b) $MnCl_2$	(c) $MgCl_2$
Nil	-	1412 (100)[d]	1284 (100)	1414 (100)
RNase A	80 µg/ml	46 (3)	23 (2)	0 (0)
DNase I	80 µg/ml	1390 (98)	1400 (109)	1387 (98)
PPi	11 mM	95 (7)	36 (3)	14 (1)
Pi	2 mM	N.D.	488 (38)	1441 (102)
Rifampicin	20 µg/ml	N.D.	1086 (85)	1353 (96)
α-amanitin	8 µg/ml	N.D.	1511 (118)	1530 (108)
ATA	5 µg/ml	N.D.	1208 (94)	1161 (82)

N.D.: not done.

(a) : assays were performed in standard assay mixtures which contained in addition 0.4 mM each of ATP, GTP and CTP.

(b) : assays were performed in standard assay mixtures from which $MgCl_2$ was omitted.

(c) : assays were performed in standard assay mixtures from which $MnCl_2$ was omitted.

(d) : % of control.

on the enzyme activity were tested in standard assay mixtures from which $MgCl_2$ was omitted. Results are shown in Table 4.3.

Table 4.3 Effects of various salts on the enzyme activity

The effect of these salts on the enzyme activity was tested by adding them to standard assay mixtures from which $MgCl_2$ was omitted.

Addition	Concentration (mM)	TCA-precipitable radioactivity (CPM)
Nil	-	1200
KCl	2	1198
KI	2	1195
$KHCO_3$	2	1050
NaH_2PO_4	2	416
KH_2PO_4	2	455

Sodium dihydrogen phosphate and potassium dihydrogen phosphate both inhibited to approximately the same extent, suggesting that the inhibition was not due to sodium or potassium ions. The monovalent anions Cl^- and I^- did not cause any significant inhibition at 2 mM concentration, but the divalent anion CO_3^{--} produced a slight degree of inhibition intermediate to that of monovalent (Cl^-, I^-) and trivalent (PO_4^{---}) anions. These results suggested that the inhibition resulting from the addition of 2 mM sodium dihydrogen phosphate might be due to the chelation of manganese ions by phosphate ions or to the displacement by phosphate ions of UTP or RNA from the enzyme.

4.3.9 NTP preference

It was found that the incorporation of radioactivity from a single labelled NTP was inhibited in the presence of the other three major NTPs. These results bear directly on the product characterization and, accordingly, will be presented in Chapter 5. The degree of preference exhibited by the enzyme activity for the various ribonucleoside triphosphates, however, can be regarded as a characteristic of the enzyme.

The NTP preference of the enzyme activity was studied in the standard assay mixture and in an assay mixture from which $MgCl_2$ was omitted. The complementary NTPs were absent in all assays and 0.08 mM of the unlabelled NTP corresponding to the labelled NTP was present in all assays. Results are shown in Table 4.4.

Table 4.4 Incorporation of a ^{3}H-NMP in the absence of the complementary NTPs

^{3}H-NTP	Specific activity (Ci/mmol)	Amount added (μCi)	pmoles NMP incorporated	
			($MnCl_2$ + $MgCl_2$)	($MnCl_2$)
ATP	24	5	16.19	N.D.
		2	N.D.	17.66
GTP	10.5	5	2.31	N.D.
		1	N.D.	0
CTP	21	5	6.72	N.D.
		1	N.D.	2.35
UTP	45	5	5.44	6.58

N.D.: not done

The phosphocellulose enzyme fraction was capable of promoting the incorporation of all four major NMPs in the presence of both $MnCl_2$ and $MgCl_2$. These NMPs were, however, incorporated to different extents. The preferred NTP was ATP, followed by CTP and UTP. GMP was also incorporated to a slight extent. In the presence of $MnCl_2$ as the sole source of divalent cations, the order of NTP preference was ATP > UTP > CTP. No GMP appeared to be incorporated under these conditions.

^{3}H-UDP, instead of ^{3}H-UTP, was added to standard assay mixtures from which either $MgCl_2$ or $MnCl_2$ was omitted, in order to determine if it could be utilized as a substrate by the enzyme activity. Two µCi (22 Ci/mmol) ^{3}H-UDP were added, as well as 0.08 mM unlabelled UDP. The control mixture contained 5 µCi (45 Ci/mmol) ^{3}H-UTP and 0.08 mM unlabelled UTP. Results are shown in Table 4.5.

Table 4.5 ^{3}H-UDP as substrate

Labelled substrate	pmoles UMP incorporated ($MnCl_2$)	($MgCl_2$)
^{3}H-UTP	4.65	7.15
^{3}H-UDP	0	0

^{3}H-UDP could not be used as a substrate by the enzyme activity under the conditions used, demonstrating that no detectable amount of polynucleotide phosphorylase was present in the phosphocellulose enzyme fraction.

4.3.10 RNA specificity

Various polyribonucleotides were assayed in standard assay mixtures from which either $MgCl_2$ or $MnCl_2$ was omitted, in order to determine if the enzyme activity showed any "preference" for a particular class of RNA. The polyribonucleotides assayed were total nuclear RNA (rat liver), high-molecular-weight nuclear RNA (rat liver), low-molecular-weight nuclear RNA (rat liver), 4S RNA (rat liver), 5.8S RNA (rat liver),"9S" RNA (rabbit reticulocyte), 18S rRNA (rat liver), 28S rRNA (rabbit reticulocyte), poly(A), poly(I), poly(C), poly(U) and poly(A,C) (1:1 random copolymer). Undenatured calf thymus DNA, a gift from Dr. Naora, was also assayed for its ability to stimulate UMP incorporation. Results are shown in Table 4.6.

DNA caused little or no stimulation of the enzyme activity, further supporting the suggestion that the reaction was DNA-independent.

The enzyme activity exhibited no marked RNA specificity, in that a great variety of RNA species, and even synthetic ribopolymers, could stimulate UMP incorporation. On a microgram basis, certain RNAs (eg. 5.8S RNA) appeared to be the favoured templates/primers. An examination of the results on the basis of the number of moles of RNA added, however, revealed that no single RNA species was favoured as template/primer by the enzyme activity. This examination is the subject of Section 5.3.3.

Table 4.6 RNA specificity.

Nucleic acid	Amount added (µg)	TCA-precipitable radioactivity (CPM) per µg added[1]	
		($MnCl_2$)	($MgCl_2$)
4S RNA	6.78	90	121
5.8S RNA	6.46	198	179
"9S" RNA	6.71	150	156
18S rRNA	6.92	49	5
28S rRNA	5.82	35	3
LMW nRNA	5.13	106	77
HMW nRNA	6.15	49	24
nRNA	6.40	102	75
DNA	6.71	40	0
poly(A)	6.09	71	121
poly(I)	6.52	18	281
poly(C)	6.50	64	61
poly(U)	7.08	114	120
poly(A,C)	6.78	22	70

[1] These calculations were performed with the values of net incorporation obtained, i.e. after the endogenous incorporation of 624 CPM ($MnCl_2$) or 1033 CPM ($MgCl_2$) was subtracted from the results.

4.4 Discussion

The results presented in this chapter are summarized in Table 4.7. The table is then followed by a discussion of the results.

Table 4.7 Summary of the results obtained in the presence of (a) $MnCl_2$ + $MgCl_2$, (b) $MnCl_2$ only and (c) $MgCl_2$ only.

Experiment	Results		
	(a) $MnCl_2$ + $MgCl_2$	(b) $MnCl_2$ only	(c) $MgCl_2$ only
1. Time course	Net UMP incorporation during the first 90 minutes of incubation	Net UMP incorporation during the first 30 minutes of incubation	Net UMP incorporation during the first 20 minutes of incubation
2. pH optimum	pH 7.8	Major optimum at pH 7.8; minor optimum at pH 7.3	Major optimum at pH 7.8; minor optimum at pH 7.3
3. Effect of temperature of incubation	N.D.	Enzymatic reaction at pH 7.8; artefact at pH 7.3	Enzymatic reaction at pH 7.8
4. Optimum temperature	N.D.	30-35°C	30-35°C
5. Effect of monovalent cations	Both ammonium and potassium ions inhibited	N.D.	N.D.
6. Effect of divalent cations	Effects of $MnCl_2$ and $MgCl_2$ not quite additive	Optimum at 1.3 mM favoured AMP incorporation; optimum at 3.9 mM favoured UMP incorporation	Optimum at 10 mM; at higher concentrations, TCA-precipitable radioactivity increased with increasing concentrations of $MgCl_2$
7. Effect of RNase A	Inhibited almost completely	Inhibited almost completely	Inhibited completely

Table 4.7 (continued)

Experiment	Results		
	(a) $MnCl_2$ + $MgCl_2$	(b) $MnCl_2$ only	(c) $MgCl_2$ only
8. Effect of DNase I	No inhibition	No inhibition	No inhibition
9. Effect of pyrophosphate	Very strong inhibition	Very strong inhibition	Very strong inhibition
10. Effect of orthophosphate	N.D.	Inhibited significantly	No inhibition
11. Effect of rifampicin	N.D.	No significant inhibition	No significant inhibition
12. Effect of α-amanitin	N.D.	No significant inhibition	No significant inhibition
13. Effect of ATA	N.D.	No significant inhibition	No significant inhibition
14. Pre-incubation with pronase	N.D.	Completely eliminated enzyme activity	Completely eliminated enzyme activity
15. Pronase added during incubation	N.D.	Did not decrease TCA-precipitable radioactivity	Did not decrease TCA-precipitable radioactivity
16. NTP preference	ATP > CTP > UTP > GTP	ATP > UTP > CTP; no GMP incorporated	N.D.

Table 4.7 (continued)

Experiment	Results		
	(a) $MnCl_2$ + $MgCl_2$	(b) $MnCl_2$ only	(c) $MgCl_2$ only
17. ^{3}H-UDP as substrate	N.D.	Not utilized	Not utilized
18. RNA specificity	N.D.	None apparent	None apparent

N.D.: not done

The results obtained in the presence of $MnCl_2$ as the sole source of divalent cations (column b, Table 4.7) and those obtained in the presence of $MgCl_2$ as the sole source of divalent cations (column c, Table 4.7) showed many similarities. Under both these conditions:

1. there appeared to be two pH optima, a major optimum at pH 7.8 and a minor optimum at pH 7.3. The optimum at pH 7.8 was shown in both cases to represent enzymatic activity.
2. the optimum temperature for activity appeared to be between 30 and 35°C.
3. RNase A and pyrophosphate severely inhibited the enzyme activity, whereas DNase I, rifampicin, α-amanitin and ATA caused no significant inhibition.
4. pre-incubation with pronase completely destroyed the enzyme activity. If pronase was added some time after the start of the incubation period, the TCA-precipitable radioactivity was not reduced in either instance.
5. ^{3}H-UDP could not be utilized as a substrate, and
6. there was no marked RNA specificity.

On the other hand, a few dissimilarities were also observed. For example, net incorporation of UMP took place during the first 30 minutes of incubation with $MnCl_2$, whereas it took place during the first 20 minutes of incubation in the presence of $MgCl_2$. Net incorporation

of UMP represented the difference between the enzyme-mediated UMP incorporation and the ribonuclease-mediated breakdown of both product and template/primer in the assay mixture. The difference in the time courses might thus simply reflect the ionic requirements of the ribonuclease(s) which were probably endogenous to the enzyme fraction from phosphocellulose chromatography. Another difference was the observation that 2 mM sodium dihydrogen phosphate inhibited the enzyme activity in the presence of $MnCl_2$, but not in the presence of $MgCl_2$.

Because of the similarities listed above, it appeared that the incorporation of ^{3}H-UMP into a TCA-precipitable product might be mediated by a single RNA-dependent enzyme activity. This activity had an absolute requirement for a divalent cation and this requirement could be met with either $MnCl_2$ or $MgCl_2$. This suggestion is supported by results obtained in the characterization of the product (Chapter 5).

The results obtained in the presence of both $MnCl_2$ and $MgCl_2$ (column a, Table 4.7) showed many similarities with those obtained in the presence of a single divalent cation (columns b and c, Table 4.7). The most notable similarities were:

1. a pH optimum in the vicinity of pH 7.8.
2. essentially no UMP incorporation in the presence of RNase A.
3. no reduction of UMP incorporation in the presence of DNase I.
4. an almost complete inhibition of enzyme activity in the presence of pyrophosphate.

The most notable difference was the duration of net UMP incorporation. The difference in the time courses, however, did not in itself demonstrate the existence of separate enzymes. In view of the similarities listed above and of characteristics shared by the products obtained under both sets of conditions (Chapter 5), it was concluded that the incorporation of UMP into TCA-precipitable material might be due to a single enzyme activity, whether $MnCl_2$, $MgCl_2$ or both were present in the assay mixture.

It is not known whether the incorporation of ^{3}H-AMP, ^{3}H-CMP and ^{3}H-GMP into TCA-precipitable material was mediated by the enzyme activity which incorporated ^{3}H-UMP or by other enzymes. The existence of a homopolymer polymerase and that of separate poly(A), poly(U), poly(G) and poly(C) polymerases in eukaryotes is considered in the final Discussion (Chapter 7) to this work, as is the existence of "replicase-like" RNA-dependent RNA polymerases.

The fact that AMP was incorporated to a greater extent than the other three major NMPs provided a strong argument for the characterization of the AMP-incorporating enzyme activity. However, ^{3}H-UTP was used as the labelled substrate. One reason for this was the existence of very many reports of poly(A) polymerases (for example, see Table 1.3). In the event that the phosphocellulose enzyme catalyzed homopolymer synthesis, assays using ^{3}H-ATP as the labelled substrate would simply re-demonstrate poly(A) synthesis in rat liver. Such work would be neither original nor very rewarding, in terms of novel information. A second and more positive reason for using ^{3}H-UTP as the labelled substrate was the growing interest in RNAs containing U-rich

segments. As mentioned in Chapter 1, such RNAs have been widely reported, but their origin and function are in many cases undetermined. The use of ^{3}H-UTP in the characterization of the enzyme activity allowed at least the possibility that some valuable insights might be gained into these problems.

The $MnCl_2$ concentration in the standard assay mixture and in "$MnCl_2$ only" mixtures was 3.9 mM. The reason is that, although peaks of enzyme activity were observed at both 1.3 mM and 3.9 mM $MnCl_2$ (Figure 4.5A), UMP incorporation was greater at 3.9 mM $MnCl_2$ than at 1.3 mM $MnCl_2$ in otherwise standard assay mixtures.

The addition of RNase A completely (column c, Table 4.2) or almost completely (columns a and b, Table 4.2) eliminated the TCA-precipitable radioactivity. This was possibly due to either or both of the following: the hydrolysis of the RNA template/primer and the hydrolysis of the labelled product immediately upon its being synthesized. These results supported the suggestion that the reaction catalyzed by the phosphocellulose enzyme was RNA-dependent.

The addition of DNase I to the assay mixtures caused no significant inhibition of the enzyme activity, thus suggesting that the latter was DNA-independent. This suggestion was supported by the observation (Table 4.6) that DNA caused little or no stimulation of the enzyme activity.

The addition of sodium pyrophosphate to the assay mixtures almost completely inhibited the enzyme activity. This suggested that

the enzyme activity might be a ribonucleotidyltransferase.

In the presence of $MgCl_2$ as the sole source of divalent cations, the addition of sodium dihydrogen phosphate (orthophosphate) caused no inhibition of the enzyme activity, thus suggesting that ^{3}H-UMP incorporation was not due to polynucleotide phosphorylase (polyribonucleotide : orthophosphate nucleotidyltransferase; E.C. No. 2.7.7.8). The reaction formula for the latter is:

$$NDP + (NMP)_n \rightleftarrows (NMP)_{n+1} + Pi$$ (Lehninger, 1975).

Polynucleotide phosphorylase is inhibited by 0.4 mM phosphate (Reid and Parsons, 1971). The observation that ^{3}H-UDP could not be used as a substrate supported the above suggestion.

Rifampicin is known to inhibit RNA synthesis in some prokaryotes (Wehrli and Staehelin, 1971) by binding to the β subunit of the DNA-dependent RNA polymerase (Lehninger, 1975). It has also been suggested that, at 10 µg/ml, it inhibits the mitochondrial DNA-dependent RNA polymerase (Reid and Parsons, 1971). The addition of rifampicin to the assay mixtures caused no significant inhibition of the enzyme activity.

Rat liver is known to contain at least two DNA-dependent RNA polymerases (Roeder and Rutter, 1969; Lewin, 1974). Polymerase I or A is a nucleolar enzyme which presumably synthesizes ribosomal RNA and its precursors. Polymerase II or B is a nucleoplasmic enzyme which presumably synthesizes messenger RNA or its precursors.

Alpha-amanitin, from the mushroom Amanita phalloides, specifically inhibits the eukaryotic polymerase II (Kedinger et al.,1970;

Lindell et al., 1970). The drug causes this inhibition by binding to the enzyme (Jacob et al., 1970; Kedinger et al., 1970; Lindell et al., 1970). Fifty percent inhibition is achieved with a dose of 0.01 µg α-amanitin/ml and maximal suppression with 0.03 µg/ml (Jacob et al., 1970). Aurintricarboxylic acid (ATA) inhibits both eukaryotic polymerase I and polymerase II. The drug inhibits polymerase II to a greater extent than polymerase I. At a concentration of 3-5 µg/ml, ATA completely inhibits polymerase II, apparently by binding to the enzyme (Akiyama et al., 1977). Neither α-amanitin nor ATA caused significant inhibition of the phosphocellulose enzyme activity. Since these drugs inhibit by binding to the enzyme (rather than by binding to the template, as does actinomycin D), there appeared to be no structural similarity between the eukaryotic DNA-dependent RNA polymerases and the enzyme activity under study. These results were rather inconclusive, however, since the active site for DNA-dependent RNA synthesis might be distinct from the active site, possibly on the same enzyme, for the observed RNA-dependent incorporation of UMP.

At concentrations greater than 10 mM $MgCl_2$ (Figure 4.5B), the TCA-precipitable radioactivity increased linearly with increasing concentrations of $MgCl_2$. Although the temperature response in the presence of $MgCl_2$ (Figure 4.3B) suggested the existence of a true enzyme activity, and although the addition of pronase after 20 minutes incubation did not reduce the TCA-precipitable radioactivity, it was possible that an artefact was present. Because of the response of the enzyme activity to increasing concentrations of $MgCl_2$, the characterization of the major reaction product containing UMP residues (Chapter 5) was mainly carried out in the presence of $MnCl_2$ rather than $MgCl_2$.

CHAPTER 5

CHARACTERIZATION OF THE PRODUCT

5.1 Introduction

The characterization of the enzyme activity which promoted the RNA-dependent incorporation of ^{3}H-UMP into TCA-precipitable material constituted only part of the objective of the present study. In order to understand the nature of the ^{3}H-UMP incorporation, it was essential that the major reaction product also be characterized.

The aim of this chapter is to investigate the nature of the major UMP-containing product. The crucial aspects to be examined would include:

(a) an investigation of whether the residues from UTP were incorporated into polyribonucleotide material,

(b) the elucidation of whether the major reaction product was a heteropolymer or homopolymer,

(c) the incorporation of ^{3}H-UMP as a function of the number of moles of RNA added to the assay mixture,

(d) the determination of whether the added RNA was used as a primer or as a template, and

(e) a determination of the length of the newly synthesized segment.

5.2 Materials and methods

5.2.1 Large-scale assay and extraction of the product RNA

A ten-fold assay mixture contained in 1.25 ml: 83 mM Tris-HCl, pH 7.8, 50 µCi (45 Ci/mmol) ^{3}H-UTP, 0.08 mM UTP, 10 µg/ml actinomycin D, 3.9 mM $MnCl_2$, 0.11 mM 2-mercaptoethanol, 632 µg "VV" protein, 578 µg "0.6 M" protein and 96 µg of 28S rRNA from mouse sarcoma 180 cells. A control mixture was prepared as above, but without the 28S rRNA. Both mixtures were incubated at 36°C for 10 minutes.

The RNA was then extracted from both mixtures by the SDS-phenol method described in Section 2.18. The ethanol precipitate was collected by centrifugation at 17000 x g for 10 minutes in the SS-34 rotor. It was then washed twice in each of 70% ethanol, absolute ethanol and ether. The RNA extracted from each mixture was dissolved in 15 µl of water.

5.2.2 Chain length determination

A ten-fold assay mixture was prepared as per Section 5.2.1, except that it contained 661 µg "VV" protein, 677 µg "0.6 M" protein and 58 µg of rabbit reticulocyte "9S" RNA. The mixture was incubated at 36°C for 10 minutes and the RNA was extracted as per Section 5.2.1. The RNA was precipitated with 5% TCA for 30 minutes on ice. It was then collected and washed as per Section 5.2.1. The RNA was dissolved in water and low-molecular-weight nuclear RNA was added to bring the RNA content to 0.2 mg in a total volume of 100 µl.

For determining the chain length a paper chromatographic system described by Hall (1963) was used. When alkali-digested RNA is subjected to this procedure, the 3'-OH terminal nucleosides are resolved, while the 5'-terminal pNps and internal Nps remain at the origin.

Twenty-five µl of 1.25 M NaOH were added, for a final concentration of 0.25 M. Alkali digestion of the RNA took place for 24 hours in a water bath at 37°C. The hydrolysate was centrifuged at 17300 x g for 10 minutes in the SS-34 rotor and a 100 µl aliquot was removed. One hundred µl of 0.25 M acetic acid were added to this aliquot, followed by 15 µl of 1.9 mM uridine (Sigma, St. Louis). This mixture was applied in 15 µl aliquots as a band 6 cm long to Whatman #1 (W. and R. Balston Ltd., England) chromatography paper (50 cm x 25 cm). The streaked material was dried between applications with hot air. As a control for the chromatography, 25 µl of 19.6 mM uridine were applied to the paper as a separate band.

The paper was placed in a Model 300 Panglas Chromatank (Shandon Scientific Company Limited, London) of dimensions 30.5 cm (length) x 20.3 cm (width) x 55.9 cm (height). The chromatogram was run by descending chromatography. The buffer consisted of the upper phase from a mixture obtained by combining 4 volumes of ethyl acetate (Ajax Chemicals, Sydney), 2 volumes of water and 1 volume of 1 propanol (Ajax Chemicals, Sydney). The chromatogram was allowed to develop for 16 hours, after which the paper was removed from the tank and allowed to air dry. The spots were located with a UV lamp. A good separation of the nucleosides from the nucleotides was obtained (14 cm)

with no evident "trailing". The spots were cut out and cut up into small pieces. The nucleotides and nucleosides were eluted from the paper with 2 ml distilled water. Aliquots from each eluate were placed in scintillation vials. Ten ml of Aquasol (New England Nuclear, Boston) were added to each vial and the radioactivity was measured in a Beckman LS-100 liquid scintillation counter.

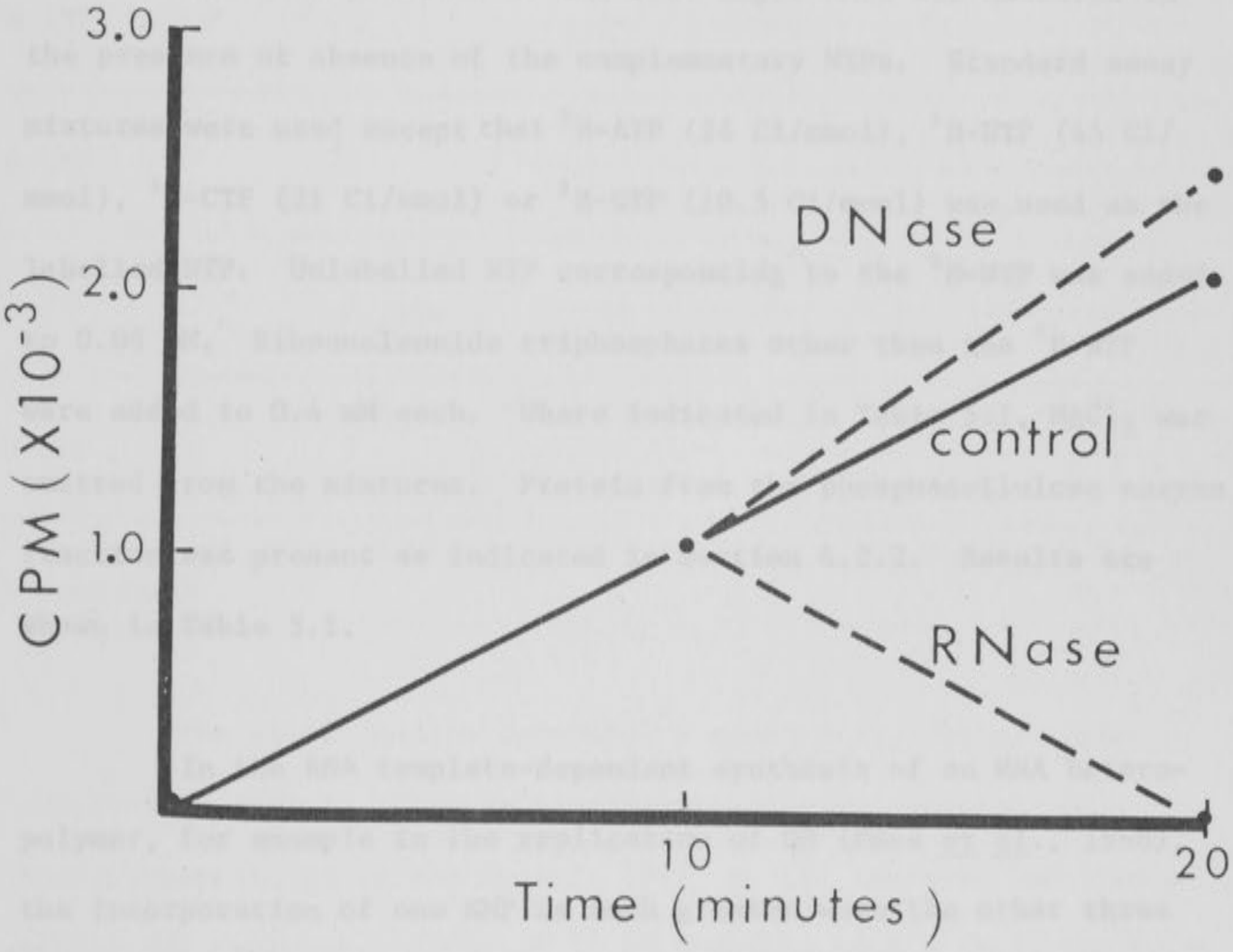
5.3 Results

5.3.1 UMP was incorporated into polyribonucleotide material

The effects of RNase A and DNase I on the labelled product were determined in standard assay mixtures (Section 2.12), except that $MgCl_2$ was omitted. Protein from the phosphocellulose enzyme fraction was added as per Section 4.2.2. After 10 minutes incubation at 36°C, RNase A and DNase I (both 80 µg/ml final concentration) were added to the assay mixtures and the incubation was continued for a further 10 minutes. Results are shown in Figure 5.1.

RNase A completely hydrolyzed the labelled product. Because RNase A is specific for pyrimidine nucleoside linkages, this result demonstrated that ^{3}H-UMP was incorporated into polyribonucleotide material. In agreement with this observation, the product was not susceptible to the action of DNase I (Figure 5.1) or pronase (Section 4.3.7). The susceptibility to RNase A also suggested that the product was probably single-stranded.

Figure 5.1 Effects of RNase A and DNase I on the labelled product.


3.0
2.0
1.0
CPM (X10-3)
DNase
control
RNase
10
20
Time (minutes)

The effects of RNase A and DNase I on the labelled product obtained in the presence of $MgCl_2$ rather than $MnCl_2$ were the same as above.

5.3.2 The major product was not a heteropolymer

The incorporation of the four major NMPs was measured in the presence or absence of the complementary NTPs. Standard assay mixtures were used except that ^{3}H-ATP (24 Ci/mmol), ^{3}H-UTP (45 Ci/mmol), ^{3}H-CTP (21 Ci/mmol) or ^{3}H-GTP (10.5 Ci/mmol) was used as the labelled NTP. Unlabelled NTP corresponding to the ^{3}H-NTP was added to 0.08 mM. Ribonucleoside triphosphates other than the ^{3}H-NTP were added to 0.4 mM each. Where indicated in Table 5.1, $MgCl_2$ was omitted from the mixtures. Protein from the phosphocellulose enzyme fraction was present as indicated in Section 4.2.2. Results are shown in Table 5.1.

In the RNA template-dependent synthesis of an RNA heteropolymer, for example in the replication of Qβ (Pace et al., 1968), the incorporation of one NMP is much greater when the other three major NTPs are present than when they are absent (cooperative effect). The synthesis of a significant amount of heteropolymer seems doubtful, however, when one NMP is incorporated to the same extent in the presence or absence of the complementary NTPs or when the incorporation of one NMP is reduced in the presence of the complementary NTPs (competitive effect).

Table 5.1 Incorporation of a ^{3}H-NMP in the presence or absence of the complementary NTPs

Labelled NTP present in assay	Amount of ^{3}H-NTP added (μCi)	Presence of unlabelled complementary NTPs	NMP incorporated (pmoles)	
			$MnCl_2$ + $MgCl_2$	$MnCl_2$
^{3}H-ATP	5	-	16.19	17.66
		+	4.26	1.46
^{3}H-UTP	5	-	5.44	6.58
		+	5.09	0.79
^{3}H-CTP	5	-	6.72	N.D.
		+	1.19	N.D.
^{3}H-GTP	5	-	2.31	N.D.
		+	1.84	N.D.

N.D.: not done

The above results indicated a competitive rather than cooperative effect. This is consistent with the synthesis of a homopolymer (Longacre and Rutter, 1977) or the terminal addition of single NMP residues. Although it is possible that a small amount of RNA-templated heteropolymer synthesis took place in the presence of all four NTPs, this was clearly not the major reaction catalyzed by the enzyme preparation under study. The major reaction product therefore may be a homopolymer segment attached to the primer RNA. The possibility that the RNA-primed synthesis of sequences containing more than one major NMP might take place in the presence of all four major NTPs cannot be ruled out.

5.3.3 ^{3}H-UMP incorporation as a function of moles of RNA present

It was shown in Section 4.3.10 that various RNAs could stimulate UMP incorporation. The addition of certain RNAs (eg. 5.8S RNA) resulted in greater TCA-precipitable radioactivity (CPM) per µg RNA added than that of other RNAs (eg. 18S or 28S rRNA). In the present section, the results of Table 4.6 are evaluated on the basis of the number of moles of each RNA species added to the assay mixture.

The RNAs resulting in the highest values of CPM/µg were from relatively small size classes, eg. 5.8S and "9S". RNAs of greater size, eg. 18S and 28S, resulted in lower values of CPM/µg. On a molar basis, however, the low-molecular-weight RNAs were not more active than the high-molecular-weight RNAs. This observation suggested that the amount of TCA-precipitable radioactivity obtained was dependent upon the number of moles of RNA added rather than on the species of RNA added.

The values of CPM/µg obtained with RNAs of known size are reproduced in Table 5.2.

Table 5.2 TCA-precipitable radioactivity (CPM) per µg of RNA vs. relative number of moles of RNA

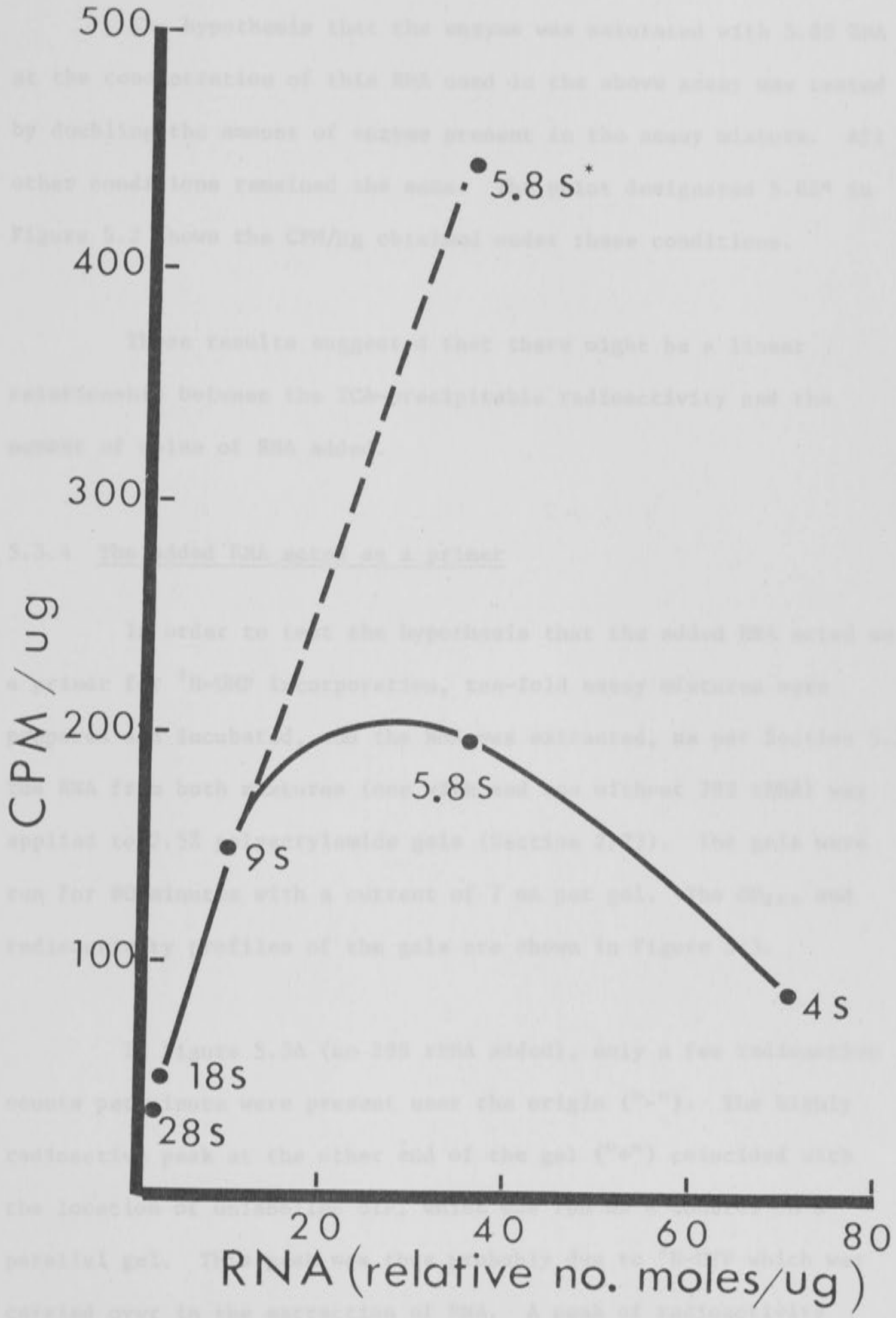
RNA	Approximate nucleotide length	Relative number of moles per µg (a)	CPM/µg (b)	CPM/mole (b)/(a)
4S	80	70.75	90	1.3
5.8S	160	35.38	198	5.6
"9S"	600	9.43	150	15.9
18S	2000	2.83	49	17.3
28S[1]	5500 + 160	2	35	17.5

[1] This 28S rRNA is the *in vivo* hybrid of one 28S rRNA molecule with one 5.8S RNA molecule. The nucleotide lengths of these RNAs are 5500 (Lewin, 1974) and 160, respectively. The value of 2 moles/ µg for 28S rRNA (third column) was arbitrarily chosen as a reference in these calculations because this undenatured 28S rRNA is a 1:1 hybrid of 28S and 5.8S RNA.

The results from Table 5.2 are plotted in Figure 5.2.

A linear relationship appeared to hold between the TCA-precipitable radioactivity obtained and the number of moles of RNA added to the assay mixture, at least for 28S, 18S and "9S" RNA. The value of CPM/µg obtained with 5.8S RNA was not on the straight line joining the values obtained with the larger RNAs. One possible explanation for this observation was that saturation of the enzyme with 5.8S RNA was reached at a lower mass of 5.8S RNA than that used in the assay. This explanation might also apply to the results obtained with 4S RNA. In addition, it is known that 4S RNA extracted from living tissue is mostly aminoacylated. This would make the 3'-ends of these molecules unsuitable as priming termini.

Figure 5.2 Relationship between ^{3}H-UMP incorporation (CPM/µg) and moles of RNA in the presence of $MnCl_2$ as the sole source of divalent cations. The point designated 5.8S* shows the effect of doubling the amount of enzyme added to the assay mixture.


500
400
300
200
100
CPM / ug
5.8 s*
9s
5.8 s
4 s
18s
28s
20
40
60
80
RNA (relative no. moles/ug)

The hypothesis that the enzyme was saturated with 5.8S RNA at the concentration of this RNA used in the above assay was tested by doubling the amount of enzyme present in the assay mixture. All other conditions remained the same. The point designated 5.8S* in Figure 5.2 shows the CPM/µg obtained under these conditions.

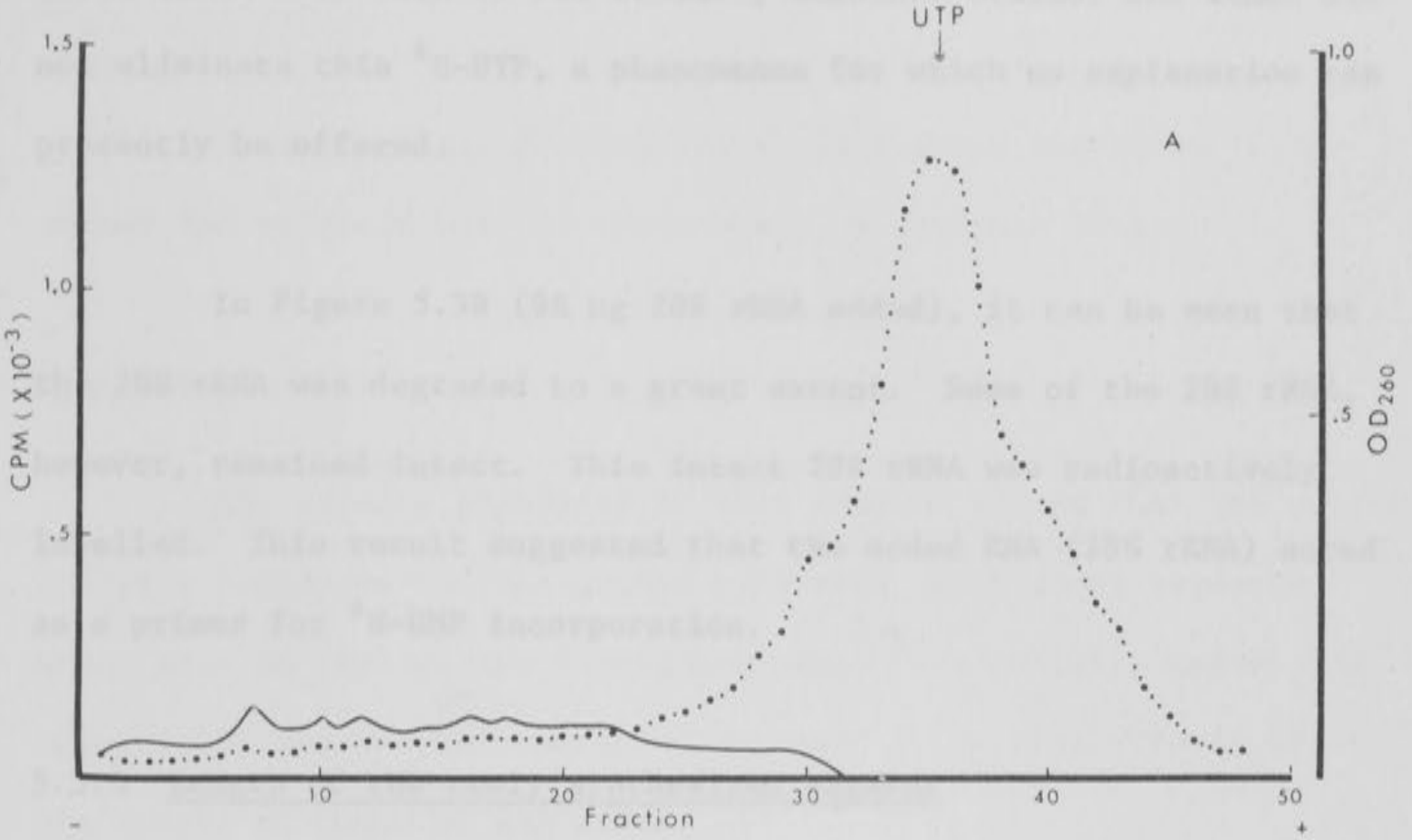
These results suggested that there might be a linear relationship between the TCA-precipitable radioactivity and the number of moles of RNA added.

5.3.4 The added RNA acted as a primer

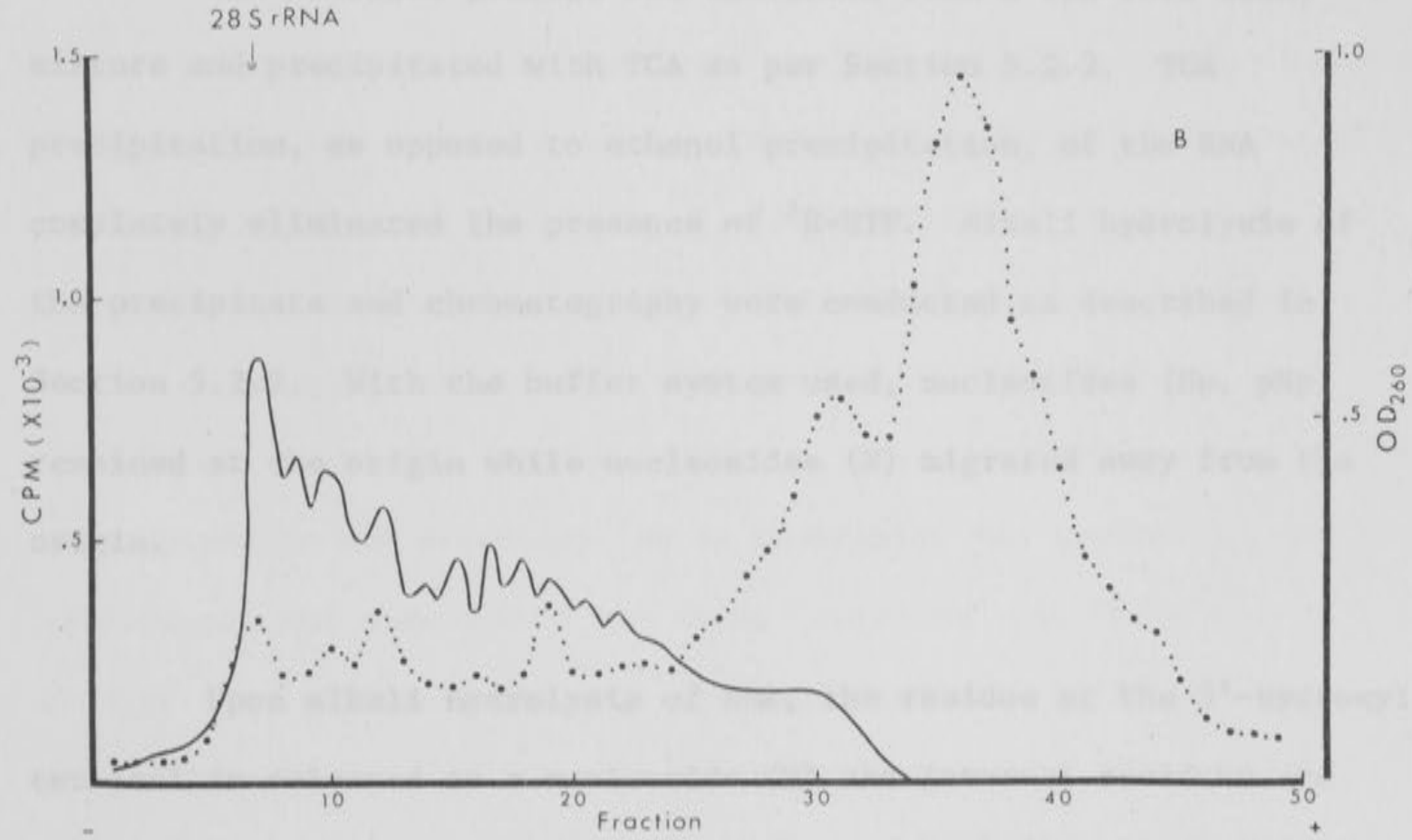
In order to test the hypothesis that the added RNA acted as a primer for ^{3}H-UMP incorporation, ten-fold assay mixtures were prepared and incubated, and the RNA was extracted, as per Section 5.2.1. The RNA from both mixtures (one with and one without 28S rRNA) was applied to 2.5% polyacrylamide gels (Section 2.23). The gels were run for 80 minutes with a current of 7 mA per gel. The OD_{260} and radioactivity profiles of the gels are shown in Figure 5.3.

In Figure 5.3A (no 28S rRNA added), only a few radioactive counts per minute were present near the origin ("-"). The highly radioactive peak at the other end of the gel ("+") coincided with the location of unlabelled UTP, which was run as a control on a parallel gel. This peak was thus probably due to ^{3}H-UTP which was carried over in the extraction of RNA. A peak of radioactivity coinciding with the location of UTP was always observed on polyacrylamide gels when the RNA extracted from the assay mixture was

Figure 5.3 Polyacrylamide gel (2.5%) electrophoresis of RNAs extracted from ten-fold assay mixtures to which were added 0 (A) or 96 µg (B) of 28S rRNA from mouse sarcoma 180 cells. The solid lines show the OD_{260} profile and the dotted lines show the radioactivity profile (large circles indicate actual CPM). The location of unlabelled UTP and 28S rRNA markers is indicated by arrows.


UTP
A
1.5
1.0
.5
CPM (X10⁻³)
1.0
.5
OD260
10
20
30
40
50
Fraction
-
+

28 S rRNA


1.5
1.0
.5
CPM (X10⁻³)
B
1.0
.5
OD260
10
20
30
40
50
Fraction
-
+

precipitated with ethanol. Washing the ethanol-precipitated RNA three times with each of 70% ethanol, absolute ethanol and ether did not eliminate this ^{3}H-UTP, a phenomenon for which no explanation can presently be offered.

In Figure 5.3B (96 µg 28S rRNA added), it can be seen that the 28S rRNA was degraded to a great extent. Some of the 28S rRNA, however, remained intact. This intact 28S rRNA was radioactively labelled. This result suggested that the added RNA (28S rRNA) acted as a primer for ^{3}H-UMP incorporation.

5.3.5 Length of the newly synthesized segment

The labelled product was extracted from a ten-fold assay mixture and precipitated with TCA as per Section 5.2.2. TCA precipitation, as opposed to ethanol precipitation, of the RNA completely eliminated the presence of ^{3}H-UTP. Alkali hydrolysis of the precipitate and chromatography were conducted as described in Section 5.2.2. With the buffer system used, nucleotides (Np, pNp) remained at the origin while nucleosides (N) migrated away from the origin.

Upon alkali hydrolysis of RNA, the residue at the 3'-hydroxyl terminal is released as a nucleoside (N) and internal residues are released as nucleoside 2'- and 3'-phosphates (Np) (Brown and Todd, 1955). The amount of radioactive label remaining at the origin, and therefore present in nucleotides, was 5388 CPM. The amount of radioactive label migrating away from the origin, and therefore

present in nucleosides, was 678 CPM. The ratio of radioactivity in Np to that in N was therefore 7.95:1.

Consequently, the oligo(U) segment which was added to the primer RNA molecule was, on the average, 8 residues long.

5.4 Discussion

The results presented in this chapter showed that the enzyme activity catalyzed the RNA-primed synthesis of oligo(U) segments which were on the average 8 residues long. The activity had no RNA specificity and UMP incorporation appeared to be proportional to the number of moles of RNA added.

The meaning of a "template" nucleic acid is generally taken to be as follows: it is a polynucleotide which is used as a "mould" or "template" for the synthesis of a polynucleotide product of complementary nucleotide sequence. The use of the term "primer", on the other hand, can lead to some confusion. The term has been used in one sense (eg. Krakow and Ochoa, 1963) to indicate that a polynucleotide was necessary for or stimulated RNA synthesis. In this sense, the meanings of the terms "template" and "primer" overlap. In another sense (eg. Boyd and Fitschen, 1975), the term "primer" is used in contrast to the term "template", to indicate a polynucleotide which provides an end, or growing point, to which additional nucleotide residues can be added by a polymerase. Recent usage favours this latter meaning (Lehninger, 1975). The terms "primer" and "primer-dependent" are used in this work in contrast to "template" and "template-dependent".

Several of the previous results (see below) suggested that the RNA added to the assay mixture acted as a primer rather than as a template. These are the observations that:

(a) the major reaction was not an RNA-templated heteropolymer synthesis (Section 5.3.2),

(b) a wide variety of RNA species could stimulate ^{3}H-UMP incorporation (Section 4.3.10),

(c) poly(U) could stimulate UMP incorporation, and that

(d) ^{3}H-UMP incorporation was dependent on the number of moles, rather than on the species, of RNA added.

The observation that the 28S rRNA added to the assay mixture became radioactively labelled (Section 5.3.4) supported this suggestion.

A model was suggested in Section 3.1.4 whereby the added RNAs, instead of acting as primers for UMP incorporation, competed with endogenous RNA for endogenous ribonuclease(s). According to this model, the addition of RNAs would result in a decreased hydrolysis of the endogenous RNAs and lead to greater "endogenous RNA-dependent" UMP incorporation. The demonstration that the added RNA became labelled (Section 5.3.4) invalidated this model; it appeared that the exogenous RNA was directly used as a primer.

The relevance of this _in vitro_ RNA-primed synthesis of oligo(U) segments to _in vivo_ conditions may be queried. Boyd and Fitschen (1975) reported the existence of an actinomycin D-insensitive,

ribosome-bound, RNA-dependent poly(U) and poly(C) polymerase in immature chicken erythrocytes. A comparison of the sedimentation properties and UMP/uridine ratio of the product synthesized *in vitro* from ^{3}H-UTP by this enzyme activity, and those of the RNA labelled in the presence of actinomycin D and ^{3}H-uridine in intact cells showed a close similarity (Boyd *et al.*, 1977). This strongly suggested that the labelled RNA synthesized *in vivo* in the presence of actinomycin D might be the product of the ribosome-associated terminal ribonucleotidyl transferase activity which they observed *in vitro*. It is presently not known if the enzyme activity characterized in the present work catalyzes the same reaction *in vivo*. It is quite possible, however, that it does. The addition of oligo(U) sequences to RNA molecules might be an important post-transcriptional event.

The enzyme activity under study may be active at several levels in the physiology of the cell. This is suggested by its lack of RNA specificity and by the observation that it has minimal ionic requirements (no monovalent cation is needed and only one of Mn^{++} and Mg^{++} is required).

CHAPTER 6

RELATIONSHIP OF THE RNA-DEPENDENT URIDYLTRANSFERASE TO DNA-DEPENDENT RNA POLYMERASE ACTIVITIES

6.1 Introduction

The phosphocellulose enzyme fraction was seen to contain an RNA-dependent enzyme activity which promoted the incorporation of nucleotide residues from ^{3}H-UTP into TCA-precipitable material. The object of this chapter is to study the enzyme activity in terms of a possible relationship to DNA-dependent RNA polymerase activities.

It is now well recognized that enzymes can have multiple activities. In the field of nucleic acid synthesis, enzymes from E. coli offer some examples. The E. coli DNA polymerase I (deoxynucleosidetriphosphate : DNA deoxynucleotidyltransferase; E.C. No. 2.7.7.7) consists of a single polypeptide chain (mol wt 109,000) which catalyzes not only extension of polydeoxyribonucleotides from the 3'-OH end group but also exonucleolytic degradation of polydeoxyribonucleotides from both the 3'-OH end group and from the 5'-end group (Klett et al., 1968; Deutscher and Kornberg, 1969). Controlled proteolytic cleavage of the enzyme with subtilisin yields a small fragment (mol wt 36,000) with 5' → 3' exonuclease activity and a large fragment (mol wt 76,000) with both polymerase and 3' → 5' exonuclease functions (Klenow and Overgaard-Hansen, 1970; Klenow et al., 1971; Setlow and Kornberg, 1972). Gulati et al. (1974) have also described conditions under which E. coli DNA polymerase I can function in vitro as an RNA-dependent DNA polymerase.

Bernard *et al*. (1977) have shown that the *E. coli* DNA-dependent RNA polymerase, which is thought to transcribe the *E. coli* genome *in vivo*, can synthesize *in vitro* complementary RNA (cRNA) directly on rRNA and mRNA templates. The conditions for this synthesis included the presence of Mn^{++} and relatively high substrate and enzyme concentrations.

Several other reports can also be found in the literature concerning the ability of DNA-dependent RNA polymerases (nucleosidase-triphosphate : RNA nucleotidyltransferases; E.C. No. 2.7.7.6) to catalyze, under certain conditions, the RNA-dependent incorporation of nucleotide residues from one or several ribonucleoside triphosphates into a TCA-precipitable product. Niyogi and Stevens (1965a,b), for example, reported that the RNA polymerase from *E. coli* could utilize synthetic polyribonucleotides as templates for the synthesis of complementary polyribonucleotides. The mechanism of polyadenylate-polyuridylate synthesis by the *E. coli* RNA polymerase holoenzyme II in the absence of template DNA was studied by Iwakura (1976). The *Azotobacter vinelandii* RNA polymerase was shown by Krakow and Ochoa (1963) to use poly(A), poly(U) and poly(C) for the incorporation of the complementary nucleotides. In still another bacterial system, *Micrococcus luteus* (formerly *M. lysodeikticus*), the RNA polymerase was shown to catalyze RNA-primed RNA synthesis (Fox *et al*., 1964).

This phenomenon is apparently not restricted to prokaryotes. Ballard and Williams-Ashman (1966) reported that a soluble form of DNA-dependent RNA polymerase from rat testes could catalyze the synthesis of complementary polyribonucleotides in the presence of

various homopolymers. Sasaki et al. (1974a,b) reported that the DNA-dependent RNA polymerase I from cauliflower (Brassica oleracea var. botrytis) could use homopolyribonucleotides as templates for the synthesis of the complementary purine (but not pyrimidine) polyribonucleotides. These reports deal mainly with RNA-templated homopolymer synthesis, whereas the reaction under study is an RNA-primed oligo(U) synthesis. The reports do illustrate, however, the fact that DNA-dependent RNA polymerases can, at least in vitro, catalyze more than one reaction. The report of Rose and Jacob (1974) bears more directly on the problem at hand. They mention that while fresh preparations of RNA polymerase from rat liver could not promote the RNA-primed synthesis of poly(A), the incubation of RNA polymerase II at 37°C resulted in its conversion to poly(A) polymerase.

The possibility arose that the RNA-dependent activity observed in the phosphocellulose enzyme fraction might be due to an enzyme which was normally recognized as being DNA-dependent. Earlier studies using known inhibitors of DNA-dependent RNA synthesis (Section 4.3.8) showed that rifampicin, α-amanitin and ATA caused no significant inhibition of the RNA-dependent activity. Native calf thymus DNA only slightly stimulated the activity in the presence of $MnCl_2$, and it did not stimulate the activity at all in the presence of $MgCl_2$ (Section 4.3.10). However, the conditions used in these assays were optimal for RNA-dependent activity; they were not necessarily favourable for DNA-dependent RNA synthesis, specially since the standard assay mixture contained 10 µg/ml actinomycin D. A more thorough investigation was required to assess the possibility that DNA-dependent RNA polymerase activities were present in the

enzyme fraction under study. Ion-exchange chromatography on DEAE-Sephadex appeared to be a suitable method to achieve this end.

Since Roeder and Rutter (1969) demonstrated the existence of two distinct DNA-dependent RNA polymerases from rat liver nuclei and three from developing sea urchin embryos by DEAE-Sephadex chromatography, this technique has been used extensively for the preparation of RNA polymerases (eg. Jacob, 1973; Rose and Jacob, 1974; Sasaki et al., 1974a,b; Longacre and Rutter, 1977).

It appeared that the "0.6 M" fraction from phosphocellulose chromatography might contain the RNA-dependent activity, while the "VV" fraction might contain some putative stimulatory "factor". The ion-exchange chromatography on DEAE-Sephadex of the "0.6 M" fraction and the monitoring of the eluate for both (a) RNA-dependent activity and (b) DNA-dependent RNA polymerase activity might yield some insight into the possible relationship between the RNA-primed uridyl-transferase and DNA-dependent RNA polymerase activities.

6.2 Materials and methods

6.2.1 DEAE-Sephadex chromatography

The method used was that of Roeder and Rutter (1969). Six mg of "0.6 M" protein in 0.3 ml of TM-25 buffer [0.05 M Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 25% (v/v) glycerol] containing 0.05 M ammonium sulfate were applied to a DEAE-Sephadex A-25 column of dimensions 1.5 cm (diameter) x 8 cm (height). The column had previously been equilibrated with 0.05 M ammonium

sulfate in TM-25. After washing with 0.05 M ammonium sulfate in TM-25 (10 ml) and 0.1 M ammonium sulfate in TM-25 (10 ml), a linear gradient (40 ml total volume) of 0.1-0.5 M ammonium sulfate in TM-25 was applied. The eluate was collected in 1 ml fractions with an Isco "Golden Retriever" fraction collector. The ammonium sulfate concentration of the eluate was monitored with a PW 9501 conductivity meter (Philips, Holland). The OD_{280} of the eluate was monitored throughout with an Isco UA-5 absorbance monitor.

The relationship between electrical conductivity, in millimhos ($m\Omega^{-1}$), and ammonium sulfate concentration (M) was determined using the following standards: 0.05 M, 0.1 M and 0.5 M ammonium sulfate in TM-25. These ammonium sulfate concentrations gave readings on the conductivity meter of 2.09, 3.25 and 12.10 $m\Omega^{-1}$, respectively. The straight line thus obtained, relating conductivity to ammonium sulfate concentration, was used as a standard curve for the conversion of the electrical conductivity in a fraction of the eluate to its corresponding ammonium sulfate concentration. The ammonium sulfate concentration of the eluate could thus be monitored throughout the chromatography.

6.2.2 Method of assay for RNA-dependent oligo(U) polymerase activity in the DEAE-Sephadex eluate

The method of assay was as given in Section 2.12. Standard assay mixtures were used, except that only 4 µg nRNA were added. Thirty µl from each fraction of the eluate to be assayed were added to the assay mixtures, which were incubated for 10 minutes at 36°C.

No protein from the stimulatory "VV" fraction from phosphocellulose chromatography was added.

6.2.3 Method of assay for DNA-dependent RNA polymerase activity in the DEAE-Sephadex eluate

The assay mixture contained the same materials as that of Roeder and Rutter (1969), but the amounts and concentrations differed slightly. In the present work, these were: 32 mM Tris-HCl, pH 7.9, 0.9 mM $MnCl_2$, 4.6 mM KCl, 0.9 mM 2-mercaptoethanol, 0.4 µmole NaF, 0.3 µmole phosphoenolpyruvate 1.4 µg pyruvate kinase, 11.5 µg native calf thymus DNA, 0.23 mM each of ATP, GTP and CTP, 0.05 mM unlabelled UTP, 5 µCi (50 Ci/mmol) ^{3}H-UTP and 50 µl from the DEAE-Sephadex fraction. The total volume was 0.125 ml. Incubation was for 10 minutes at 36°C.

6.3 Results

6.3.1 DEAE-Sephadex chromatography

Chromatography on DEAE-Sephadex A-25 was described in Section 6.2.1. A typical OD_{280} elution profile is shown in Figure 6.1.

Various fractions were assayed for RNA-dependent oligo(U) polymerase activity as per Section 6.2.2. The TCA-precipitable radioactivity (CPM) obtained with each fraction is plotted in Figure 6.2A against the concentration of ammonium sulfate present in that fraction.

Figure 6.1 DEAE-Sephadex A-25 chromatography of the "0.6 M" fraction from phosphocellulose chromatography. The OD_{280} of the eluate is shown by the solid line and the concentration of ammonium sulfate is shown by the dotted line.

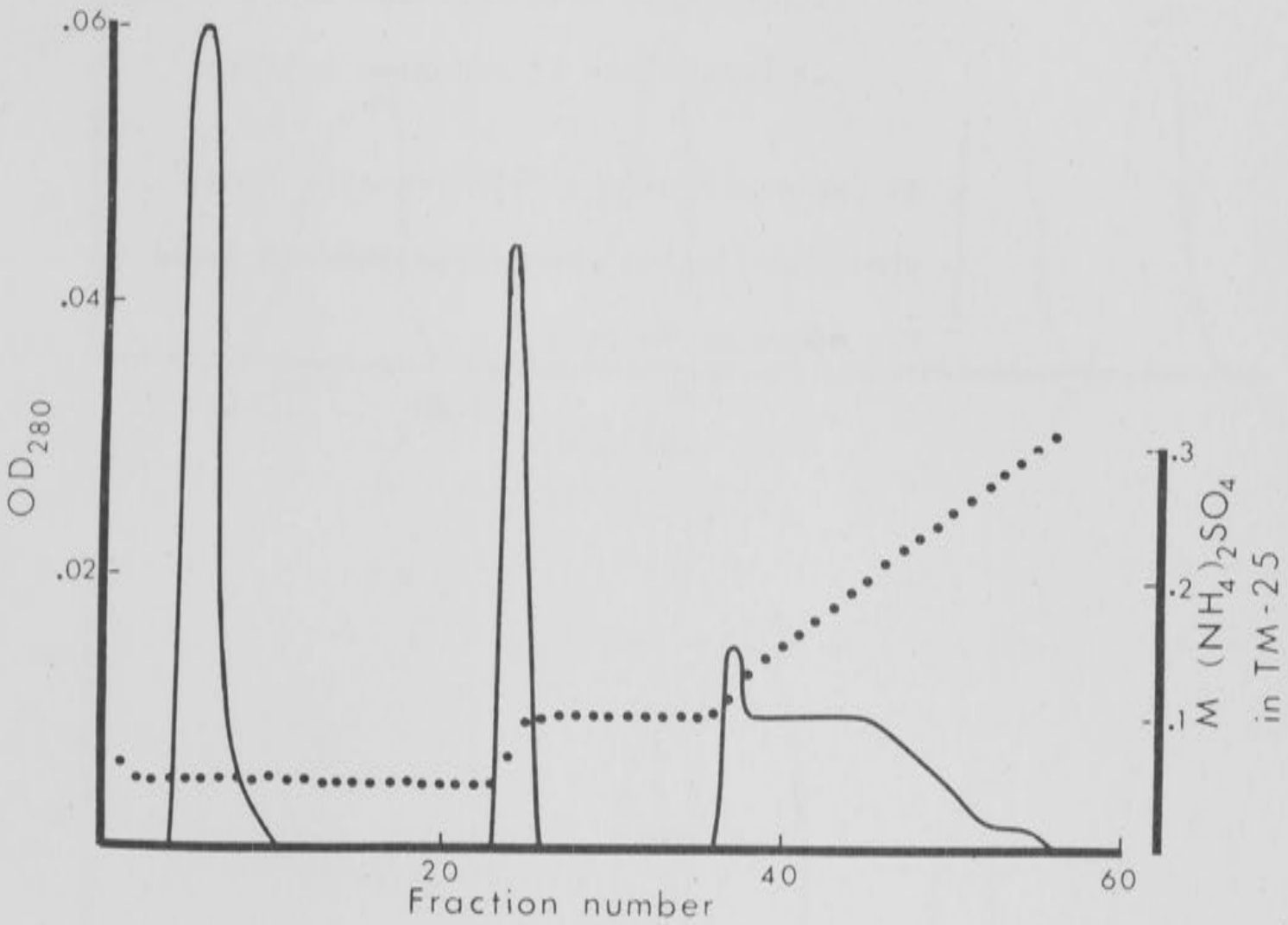
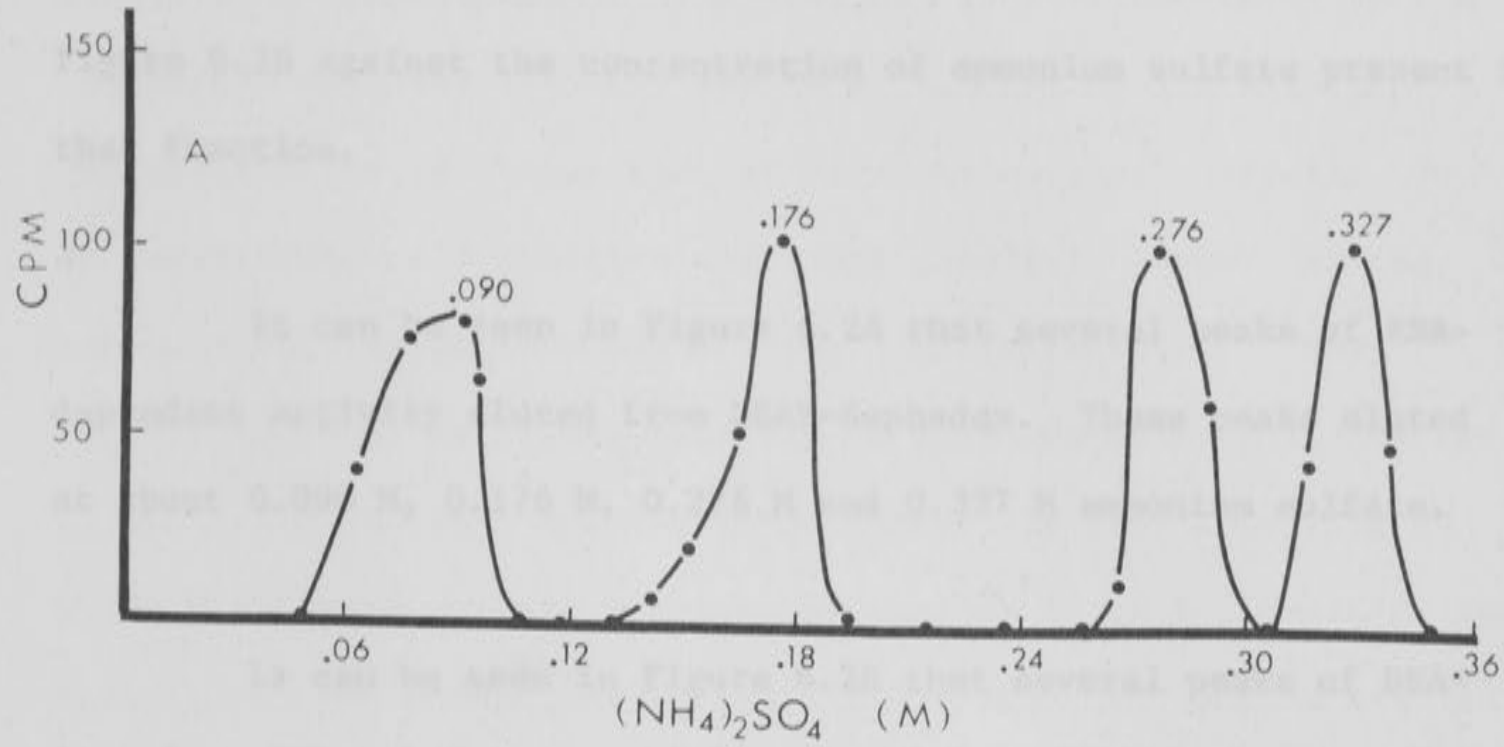
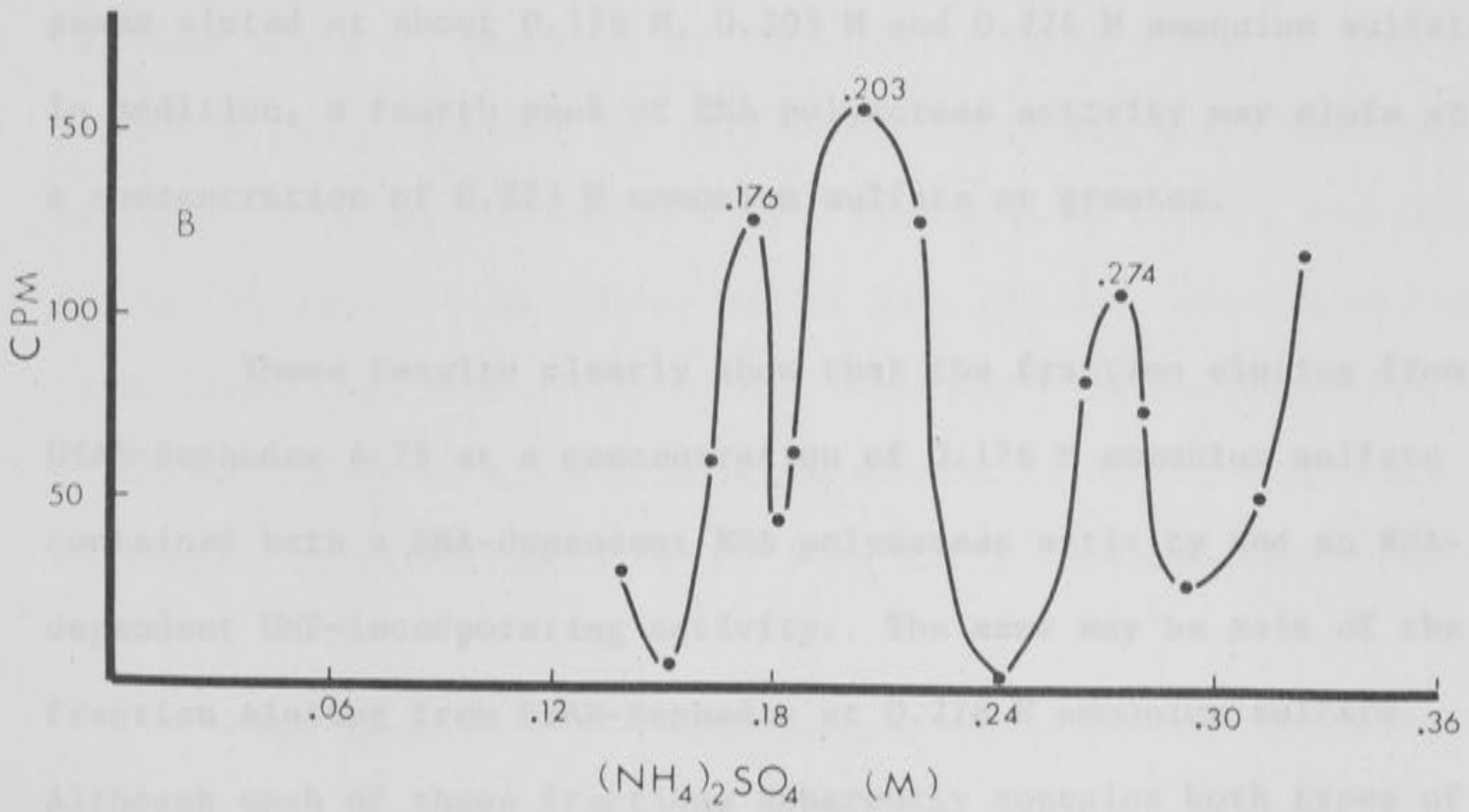

.06
.04
.02
OD280
20
40
60
Fraction number
.3
.2
.1
M (NH4)2SO4
in TM-25

Figure 6.2 A. RNA-dependent UMP-incorporating activity eluting from DEAE-Sephadex at different concentrations of ammonium sulfate.

B. DNA-dependent RNA polymerase activity eluting from DEAE-Sephadex at different concentrations of ammonium sulfate.

No protein from the "VV" fraction from phosphocellulose chromatography was added to the assay mixtures.


150
100
50
CPM
A
.090
.176
.276
.327
.06
.12
.18
.24
.30
.36
$(NH_4)_2SO_4$ (M)


150
100
50
CPM
B
.176
.203
.274
.06
.12
.18
.24
.30
.36
$(NH_4)_2SO_4$ (M)

Fractions were also assayed for the presence of DNA-dependent RNA polymerase activity as per Section 6.2.3. The TCA-precipitable radioactivity (CPM) obtained with each fraction is plotted in Figure 6.2B against the concentration of ammonium sulfate present in that fraction.

It can be seen in Figure 6.2A that several peaks of RNA-dependent activity eluted from DEAE-Sephadex. These peaks eluted at about 0.090 M, 0.176 M, 0.276 M and 0.327 M ammonium sulfate.

It can be seen in Figure 6.2B that several peaks of DNA-dependent RNA polymerase activity eluted from DEAE-Sephadex. These peaks eluted at about 0.176 M, 0.203 M and 0.274 M ammonium sulfate. In addition, a fourth peak of RNA polymerase activity may elute at a concentration of 0.323 M ammonium sulfate or greater.

These results clearly show that the fraction eluting from DEAE-Sephadex A-25 at a concentration of 0.176 M ammonium sulfate contained both a DNA-dependent RNA polymerase activity and an RNA-dependent UMP-incorporating activity. The same may be said of the fraction eluting from DEAE-Sephadex at 0.276 M ammonium sulfate. Although each of these fractions apparently contains both types of activity, it is not clear whether the DNA-dependent RNA polymerase activities eluting at 0.176 M and 0.276 M ammonium sulfate are responsible for the RNA-dependent incorporation of UMP.

6.4 Discussion

Several peaks of DNA-dependent RNA polymerase activity eluted from DEAE-Sephadex. The positive identification of an RNA polymerase requires not only the determination of its elution characteristics, but also that of other biochemical criteria, such as sensitivity to α-amanitin and other catalytic properties (eg. ammonium sulfate optimum and Mn^{++}/Mg^{++} activity ratio). The activities shown in Figure 6.2B can be only tentatively identified on the basis of their elution properties. The peak appearing at 0.274 M ammonium sulfate might be polymerase II or B. Smuckler and Tata (1972) and Roeder and Rutter (1969) found that polymerase II from rat liver eluted from DEAE-Sephadex at approximately 0.28 M ammonium sulfate. Smuckler and Tata (1972) found a peak of DNA-dependent RNA polymerase activity at approximately 0.195 M ammonium sulfate. This may correspond to the peak seen at 0.203 M ammonium sulfate in Figure 6.2B. Smuckler and Tata did not investigate this peak, other than noting that it was α-amanitin-insensitive. The peak of activity seen at 0.176 M ammonium sulfate in Figure 6.2B might correspond to polymerase I or A. This was not investigated further.

The appearance of DNA-dependent RNA polymerase activities in the DEAE-Sephadex eluate may represent leakage of nuclear enzymes during the preparation of the crude enzyme fraction. Certain workers, however, have reported evidence suggesting that DNA-dependent RNA polymerase activities may exist in the cytoplasm *in vivo* (eg. Bell and Brown, 1972; Seifart *et al.*, 1972; Seifart and Benecke, 1975; Austoker *et al.*, 1974). Whether the existence of these activities

in the cytoplasm reflects leakage or represents a form of enzyme "storage", or whether these activities possess a cytoplasmic function in vivo still remains to be elucidated.

A comparison of Figure 6.2A with Figure 6.2B shows that at least two of the peaks of RNA-dependent activity coincide with peaks of DNA-dependent activity. Clearly, the fractions eluting at 0.176 M and 0.276 M ammonium sulfate contain both a DNA-dependent RNA polymerase activity and an RNA-dependent enzyme activity which incorporated UMP into TCA-precipitable material. Whether both activities, when they are found in a single fraction, are due to the same protein has not been ascertained. The reports mentioned in Section 6.1 concerning the ability of various DNA-dependent RNA polymerases to catalyze RNA-dependent reactions suggest, however, that this might be the case.

Finally, the chromatography of the "0.6 M" fraction from phosphocellulose on DEAE-Sephadex resulted in four peaks of RNA-dependent activity. It is not known whether these four peaks catalyze the same reaction. The enzyme activity which eluted at 0.176 M ammonium sulfate may be similar to that obtained by Boyd and Fitschen (1975) from avian erythrocytes. Their activity eluted at 0.18 M ammonium sulfate and it catalyzed the RNA-primed incorporation of UMP and CMP.

CHAPTER 7

DISCUSSION

7.1 Summary

The objectives of this work were to investigate and characterize an enzyme activity from rat liver which could promote the RNA-dependent incorporation of ^{3}H-UMP from ^{3}H-UTP into TCA-precipitable material.

The method of preparing the crude enzyme fraction was improved from that of Naora (1975) in that the optimum ammonium sulfate "cut" was determined and the EDTA step eliminated. The crude fraction was shown to catalyze an RNA-dependent reaction.

The majority of the work presented in this thesis was carried out with material obtained through phosphocellulose chromatography of the crude enzyme fraction. The "VV" and "0.6 M" fractions from phosphocellulose chromatography exerted a cooperative effect on UMP incorporation. This suggested the existence of a stimulatory "factor" acting on the RNA-dependent enzyme activity. The RNA-dependence of the phosphocellulose enzyme (i.e. "VV" + "0.6 M") was demonstrated by adding increasing amounts of nRNA to the assay mixture. The phosphocellulose enzyme fraction could stimulate the incorporation of AMP, UMP and CMP. Little or no GMP incorporation took place.

The enzyme activity catalyzing the incorporation of UMP exhibited the following characteristics:

(a) two pH optima, a minor one at pH 7.3 and a major one at about pH 7.8. The optimum at pH 7.8 appeared to be due to an enzyme activity, whereas the optimum at pH 7.3 appeared to be due to an artefact.

(b) a temperature optimum between 30-35°C.

(c) no requirement for a monovalent cation.

(d) an absolute requirement for a divalent cation. This requirement could be met with either $MnCl_2$ or $MgCl_2$. The optimum $MnCl_2$ concentrations were 1.3 mM and 3.9 mM.

(e) pre-incubation with pronase completely destroyed the enzyme activity.

(f) RNase A and pyrophosphate strongly inhibited the enzyme activity but DNase I, rifampicin, α-amanitin and ATA caused no significant inhibition. Ortho-phosphate had no effect on UMP incorporation when $MgCl_2$ was the source of divalent cations, but it inhibited UMP incorporation significantly when $MnCl_2$ was the source of divalent cations. This inhibition was not a product inhibition of poly-nucleotide phosphorylase activity, since ^{3}H-UDP could not be used as a substrate by the enzyme activity. The inhibition was possibly due to the chelation of manganese ions by phosphate ions or to the displacement by phosphate ions of UTP or RNA from the enzyme.

(g) various polyribonucleotides, both naturally-occurring and synthetic, could stimulate UMP incorporation. On a microgram basis, 5.8S and "9S" RNAs stimulated the activity more than RNAs of greater size. When the data were analyzed on a molar basis, however, no single RNA species appeared to be "preferred" by the enzyme activity. The amount of UMP incorporated appeared to depend, rather, on the number of moles of RNA added to the assay mixture.

(h) more UMP was incorporated into TCA-precipitable material when ATP, GTP and CTP were absent than when they were present, suggesting that the major UMP-incorporating reaction catalyzed by the phosphocellulose enzyme was not an RNA-templated heteropolymer synthesis. Rather, it appeared to be an RNA-primed poly(U) synthesis or the terminal addition onto RNA primer molecules of single UMP residues.

Under conditions which favoured poly(U) synthesis, i.e. in the absence of ATP, GTP and CTP, the product exhibited the following characteristics:

(a) it was susceptible to the action of RNase A. This suggested that the product was polyribonucleotide material; it also suggested that the product was a single-stranded molecule.

(b) it was not susceptible to the action of DNase I or pronase. These observations agree with the suggestion that UMP was incorporated into polyribonucleotide material.

(c) UMP residues appeared to be added onto RNA primers. A primer function for the added RNA was suggested by the observations that various RNA species, including poly(U), stimulated UMP incorporation and that 28S rRNA added to the assay mixture became radioactively labelled.

(d) the average length of the newly synthesized segment appeared to be 8 nucleotide residues.

When the material contained in the "0.6 M" fraction from phosphocellulose chromatography was further fractionated through DEAE-Sephadex chromatography, several peaks of RNA-dependent UMP-incorporating activity were observed. Two of these were present in fractions which also catalyzed DNA-dependent RNA synthesis. These observations suggested that several RNA-dependent UMP-incorporating enzyme activities might be present in the "0.6 M" fraction, and also that the RNA-dependent incorporation of UMP into a TCA-precipitable product might be mediated by DNA-dependent RNA polymerases.

7.2 Possible modes of incorporation

In order to place these findings in the wider context of RNA-dependent NMP incorporation, the various ways in which NMP residues can be incorporated into TCA-precipitable material are

considered. These are as follows:

(a) a template-dependent heteropolymer synthesis,

(b) a template-dependent homopolymer synthesis,

(c) terminal addition of residues on RNA primer molecules.

7.2.1 Template-dependent heteropolymer synthesis

The synthesis of an RNA heteropolymer on a prokaryotic or eukaryotic RNA template requires the existence of an enzyme which can function in a manner analogous to the viral replicases (eg. Section 1.2.3). Although several reports can be found in the literature (Table 1.1) which suggest that such an enzyme activity might be present in "uninfected" prokaryotes and eukaryotes, no convincing demonstration of its existence has yet been published.

A replicase-like RNA-dependent RNA polymerase in "uninfected" eukaryotes would offer a means of post-transcriptional regulation of gene expression, for example by messenger RNA amplification. Several important considerations which arise in a discussion of mRNA amplification need to be mentioned.

First, the product of mRNA-templated RNA synthesis would have to be of approximately the same size as the mRNA itself. The synthesis of this product would have to be demonstrated *in vivo* as well as *in vitro*.

Second, mRNA amplification would entail two sequential RNA-templated syntheses. The enzyme would initially have to catalyze the synthesis of an RNA heteropolymer complementary to the mRNA and it would then have to synthesize an RNA heteropolymer using this complementary RNA as a template.

Third, it would have to be shown that the cells from which the putative "mRNA-amplifying" enzyme was extracted were "uninfected". This question is of fundamental importance in considering the replication of RNA molecules. The demonstration that cells do not contain RNA viral genomes (or their DNA equivalent) will likely prove very difficult. It is becoming increasingly clear that no sharp demarcation line can be drawn between the genomes of viruses and the genomes of their target cells. The phenomenon of bacterial transduction is now well recognized. The lysogenic cycle of the coliphage λ demonstrates that viral genomes can become integrated within the host cell genome without immediately apparent consequences to the host cell physiology. It also provides a mechanism whereby the nucleic acid composition of the host cell genome can undergo modifications. Modifications in the nucleic acid sequence and composition of the genome of viruses and cells are inherent to the process of evolution. Viruses are generally considered to have evolved from cell nucleic acids (Gillespie and Gallo, 1975). Although many viruses appear to bear little genetic relationship to the cells they infect, the RNA genomes of animal tumour viruses have nucleotide sequences that are similar to sequences found in the DNA of normal cells (Gillespie and Gallo, 1975). With the discovery of the

"reverse transcriptase" enzyme (Temin and Mizutani, 1970), it became apparent that the genomes of these viruses might be integrated within the host cell genome. Although the development of neoplasms is still not fully understood, it appears that the potential for neoplastic events may exist in "uninfected" animals long before any clinical symptoms become manifest. The above comments simply relate viral genomes to the genomes of prokaryotic and eukaryotic cells. They do not appear to pertain to RNA replicase-like enzymes. They do become pertinent, however, when one considers the existence of viroids. These nucleic acid segments, mentioned in Chapter 1, are known to cause certain diseases in eukaryotes (eg. potato spindle tuber). Since they do not contain a nucleic acid sequence coding for a replicase, their replication must be mediated by a cellular enzyme. It has been suggested (Siegel and Hariharasubramanian, 1974) that a replicase-like RNA-dependent RNA polymerase reported in apparently uninfected Chinese cabbage (Astier-Manifacier and Cornuet, 1971) may be responsible for the replication of such a viroid.

Since, on the one hand, viruses are still being generated from cells (Gillespie and Gallo, 1975) and since, on the other hand, viruses can become integrated into host cell genomes, there would appear to be a continuity between the evolution of viral genomes and that of host cell genomes. In the case of RNA viruses, this continuity may make it difficult to ascertain whether a putative RNA-dependent RNA polymerase from "uninfected" eukaryotes functions in the physiology of "uninfected" cells. The question of what "normal" function such an enzyme performs might even become meaningless, as

greater understanding is achieved concerning the relationship between the host cell genome and the genome of other replicating nucleic acid units.

In the present work, no evidence was obtained which suggested the existence of a replicase-like RNA-dependent RNA polymerase activity in "uninfected" rat liver. The incorporation of ^{3}H-NMPs from the corresponding ^{3}H-NTPs was not stimulated by the addition of the complementary NTPs; in most instances, it was reduced (Chapter 5). This clearly showed that the major reaction catalyzed by the phosphocellulose enzyme was not the RNA-templated synthesis of RNA heteropolymers. Rather, it appeared to be either an RNA-primed homopolymer synthesis or an RNA-primed terminal addition of single NMP residues. It is possible, however, that the presence of all four major NTPs reduced the extent of homopolymer synthesis and favoured a small amount of heteropolymer synthesis.

7.2.2 Template-dependent homopolymer synthesis

As mentioned in Sections 1.3.2 and 6.1, prokaryotic and eukaryotic DNA-dependent RNA polymerases can, under certain conditions, catalyze the RNA homopolymer-templated synthesis of the complementary RNA homopolymers. In the present work, it was found that poly(A) and poly(A)-containing RNAs (eg. "9S" RNA) could stimulate UMP incorporation (Table 4.6). However, the incorporation of UMP was even greater in the presence of poly(U) and in the presence of RNAs which are known not to contain sizeable poly(A) tracts (eg. 5.8S RNA). These results suggested that template-dependent poly(U) synthesis,

if present, was not as extensive as primer-dependent poly(U) synthesis.

7.2.3 Terminal addition of residues on RNA primer molecules

Evidence was presented in Chapter 5 concerning the priming function of RNA added to the assay mixture. It was also seen in that chapter that the various NTPs exerted a competitive rather than a cooperative effect on the incorporation of a labelled NMP. Such a competitive effect is consistent with the terminal addition of single NMP residues or of chains of NMP residues on RNA primer molecules.

Several reports are listed in Tables 1.3 to 1.6 concerning the RNA-primed incorporation of NMPs from NTPs. The reports of Wilkie and Smellie (1968a,b) and of Longacre and Rutter (1977) are most pertinent to the present work. Both of these groups found RNA-dependent enzyme activities in rat liver microsomes which could incorporate residues from ^{3}H-UTP into TCA-precipitable material. The substrate preference of their enzyme activities was similar to that of the phosphocellulose enzyme characterized in the present study: AMP from ATP was incorporated to the greatest extent, followed by UMP from UTP and CMP from CTP. Guanosine 5'-triphosphate could not be used as a substrate.

In the presence of UTP alone, the product (Chapter 5) consisted of an oligo(U) chain 8 residues in length (average) attached to the RNA primer. This agrees closely with the results of Wilkie and Smellie (1968a).

7.3 Possible functions of the rat liver uridyltransferase

Several post-transcriptional modifications of RNAs are known, although the physiological function of the modification is not always clear. One example is the terminal addition of a -CCA triplet of bases to the 3'-OH end of tRNAs. It is known, in this case, that the modification renders the tRNAs useable as substrates by the aminoacyl-tRNA synthetases. Other examples are the addition of poly(A) sequences to the 3'-ends of HnRNA and mRNA (Section 1.4.2) and the addition of 5'-terminal "caps" on mRNA (Muthukrishnan *et al.*, 1975).

The addition of an oligo(U) segment to various RNAs may similarly constitute an important post-transcriptional modification of the RNAs. The observation that many RNA species can be utilized as primers suggests that the function of the oligo(U) segment may be a relatively non-specific one, such as possibly the modification of RNA stability. On the other hand, it is possible that 3'-terminal oligo(U) segments may play different roles depending upon the RNA to which they are bound. For example, they may function in the control of mRNA translation; this is suggested by the observation that 28S, 18S and "9S" RNAs can all stimulate the enzyme activity. The oligo(U)-synthesizing activity characterized in this work may be partly responsible for the synthesis of the tcRNAs (translational control RNAs) reported by Bogdanovsky *et al.*(1973), Bester *et al.* (1975) and Lee-Huang *et al.* (1976). A further possibility is that the lack of RNA specificity observed *in vitro* may not be representative of *in vivo* conditions. It is possible that "factors" exist in the cell which confer RNA specificity upon the enzyme activity *in vivo*.

A consideration which is often neglected in discussions on eukaryotic RNA synthesis is the observation that RNA is often found *in vivo* as a constituent of ribonucleoprotein particles (RNPs). RNPs have been observed both in the nucleus (Moulé and Chauveau, 1968; Samarina *et al.*, 1968; Niessing and Sekeris, 1971; Molnar *et al.*, 1975; Kish and Pederson, 1977) and in the cytoplasm (Henshaw, 1968; Spirin, 1969; Gander *et al.*, 1973; Kish and Pederson, 1976). They may represent the true *in vivo* condition of gene transcripts. RNP particles from rat liver nuclei were reported to contain two poly(A)-synthesizing enzymatic activities as well as a ribohomopolymer synthetase which can polymerase ATP, UTP, GTP and CTP to the corresponding ribohomopolymers (Niessing and Sekeris, 1973). The synthesis of oligo(U) segments on primer RNA molecules within native RNPs *in vivo* might constitute a fundamental event in the post-transcriptional life of various RNA molecules. Further research on the nature and function of RNPs may clarify this point.

7.4 Purification of the uridyltransferase

Phosphocellulose chromatography of the crude enzyme fraction prepared from a rat liver homogenate yielded only a partially purified enzyme fraction (phosphocellulose enzyme). The results of DEAE-Sephadex chromatography (Chapter 6) indicated that several enzymes might be present in rat liver which promote the RNA-dependent incorporation of UMP (from UTP) into TCA-precipitable material. Some fractions of the DEAE-Sephadex eluate which could promote this reaction could also promote DNA-dependent RNA synthesis. It is thus possible that the RNA-dependent incorporation of UMP (from UTP) might

be due to DNA-dependent RNA polymerases. These enzymes, apart from catalyzing the transcription of DNA, can perform alternative reactions *in vitro*. These reactions, mentioned in Chapter 6, consist mainly of ribohomopolymer-templated ribohomopolymer synthesis. It has also been reported (Bernard *et al.*, 1977) that the *E. coli* RNA polymerase could synthesize short copies (2-4S) of complementary RNA (cRNA) on eukaryotic 28S, 18S and 5S RNA, as well as on hemoglobin mRNA. This synthesis took place *in vitro* under conditions of relatively high substrate and enzyme concentrations. Bernard *et al.* (1977) suggested that the reaction could be utilized as a tool for research on the localization of RNA and of the genes for various RNAs.

In the system under study, the presence of DNA-dependent RNA polymerase activities in the DEAE-Sephadex eluate might be due to leakage of enzymes from the nucleus in the preparation of the crude enzyme fraction. Alternatively, the activities may represent cytoplasmic enzymes.

7.5 Topics which merit further investigation

The present work consisted mainly of the characterization of the partially purified enzyme activity from rat liver which incorporated UMP (from UTP) into TCA-precipitable material and the characterization of the major UMP-containing product.

Results were in agreement with earlier reports on RNA-dependent UMP incorporation in rat liver (Wilkie and Smellie, 1968; Longacre and Rutter, 1977). In addition, the results of the present work

suggested that more than one RNA-dependent UMP-incorporating activity might be present and that RNA-dependent reactions might be catalyzed by DNA-dependent RNA polymerases.

Several questions which can be asked as a result of the present research are listed below.

First, is the cooperative effect observed between the "VV" and "0.6 M" fractions from phosphocellulose chromatography due to the presence in either of these fractions of a "σ-like" factor? Similar observations on the existence of factors in eukaryotes which stimulate RNA synthesis were mentioned in Section 3.2.4. It has not been elucidated, however, whether these factors, like the E. coli σ subunit, have a regulatory function.

Second, is the incorporation of AMP (from ATP) and CMP (from CTP) due to the same enzyme activity(-ies) which catalyze(s) the incorporation of UMP (from UTP)? That several enzymes may be involved in the polymerization of different NMPs is suggested by the report of Niessing (1975), who purified three distinct forms of nuclear poly(A) polymerase from rat liver nuclei as well as an enzyme system capable of synthesizing RNA-primed poly(U), poly(G) and poly(C).

Finally, can rat liver DNA-dependent RNA polymerase activities promote the RNA-primed incorporation of UMP (from UTP)? Yu (1976) has shown that ribohomopolymer polymerases contained in isolated rat liver nuclei constituted an important source of error in the radio-

active precursor incorporation studies of nuclear DNA-dependent RNA synthesis. He estimated that when [^{14}C]ATP is used as the labelled precursor, the error is as high as 35%, [^{14}C]CTP, 20%, [^{14}C]UTP or [^{14}C]GTP, 10%. It was observed, in the present study, that some fractions of the eluate from DEAE-Sephadex chromatography which could catalyze RNA-dependent UMP incorporation could also promote DNA-dependent RNA synthesis. This observation suggested that DNA-dependent RNA polymerases might also be responsible for the RNA-dependent incorporation of UMP.

The first two questions, concerning a stimulatory "factor" and the identity of the ribohomopolymer polymerases, will require that the various proteins involved be purified to a high degree. Various purification methods will have to be applied until a suitable method is found. The third question, concerning the ability of DNA-dependent RNA polymerase activities to catalyze the RNA-primed incorporation of UMP, can be answered as follows. The known DNA-dependent RNA polymerases from rat liver can be prepared through conventional techniques. The purified polymerases can then be assayed under the standard conditions (Chapter 2) for RNA-dependent UMP incorporation.

As can be seen from this thesis, the understanding of the nature and possible functions of poly(U) polymerases is just dawning. Their complete elucidation will require further research, which may reveal mechanisms of fundamental importance in the cellular metabolism of RNA.

BIBLIOGRAPHY

Adesnik, M., Salditt, M., Thomas, W. and Darnell, J.E. (1972). J. Mol. Biol., 71, 21-30.

Akiyama, S., Kuwano, M., Yamamoto, M. and Endo, H. (1977). J. Biochem., 81, 135-141.

Amano, M. (1967). Exptl. Cell Res., 46, 169-179.

Appels, R. and Williams, A.F. (1970). Biochim. Biophys. Acta, 217, 531-534.

Astier-Manifacier, S. and Cornuet, P. (1971). Biochim. Biophys. Acta, 232, 484-493.

August, J.T., Ortiz, P.J. and Hurwitz, J. (1962). J. Biol. Chem., 237, 3786-3793.

Austoker, J.L., Beebee, T.J.C., Chesterton, C.J. and Butterworth, P.H.W. (1974). Cell, 3, 227-234.

Ballard, P.L. and Williams-Ashman, H.G. (1966). J. Biol. Chem., 241, 1602-1615.

Bell, E. and Brown, J. (1972). Biochim. Biophys. Acta, 269, 237-246.

Bernard, O., Cory, S. and Adams, J.M. (1977). Biochim. Biophys. Acta, 478, 407-416.

Bester, A.J., Kennedy, D.S. and Heywood, S.M. (1975). Proc. Natl. Acad. Sci. U.S.A., 72, 1523-1527.

Bogdanovsky, D., Hermann, W. and Schapira, G. (1973). Biochem. Biophys. Res. Commun., 54, 25-32.

Boyd, C.D. and Fitschen, W. (1975). Biochem. Biophys. Res. Commun., 67, 1210-1218.

Boyd, C.D., Thompson, P. and Fitschen, W. (1977). Biochem. Biophys. Res. Commun., 78, 985-991.

Brandhorst, B.P. and McConkey, E.H. (1975). Proc. Natl. Acad. Sci. U.S.A., 72, 3580-3584.

Brawerman, G. and Diez, J. (1975). Cell, 5, 271-280.

Brishammar, S. and Juntti, N. (1975). Biochim. Biophys. Acta, 383, 351-358.

Brown, D.M. and Todd, A.R. (1955). In "The Nucleic Acids" (Chargaff, E. and Davidson, J.N., Eds.), Vol. 1, p.409, Academic Press, New York.

Burdon, R.H. and Shenkin, A. (1972). FEBS Lett., 24, 11-14.

Burdon, R.H., Shenkin, A., Douglas, J.T. and Smillie, E.J. (1977). Biochim. Biophys. Acta, 474, 254-267.

Burdon, R.H. and Smellie, R.M.S. (1961). Biochim. Biophys. Acta, 51, 153-162.

Burgess, R.R. (1969a). J. Biol. Chem., 244, 6160-6167.

Burgess, R.R. (1969b). J. Biol. Chem., 244, 6168-6176.

Burgess, R.R., Travers, A.A., Dunn, J.J. and Bautz, E.K.F. (1969). Nature, 221, 43-46.

Burkard, G. and Keller, E.B. (1974). Proc. Natl. Acad. Sci. U.S.A., 71, 389-393.

Byrnes, J.J., Downey, K.M., Jurmark, B.S. and So, A.G. (1974). Nature, 248, 687-690.

Chakravorty, A.K. and Biswas, B.B. (1965). J. Biol. Chem., 240, 4406-4413.

Clark, S., Losick, R. and Pero, J. (1974). Nature, 252, 21-24.

Colvill, A.J.E. and Terzi, M. (1968). Biochim. Biophys. Acta, 155, 394-400.

Cramer, D. (1974). Path. Microbiol., 40, 139.

Crestfield, A.M., Smith, K.C. and Allen, F.W. (1955). J. Biol. Chem., 216, 185-193.

Darnell, J.E., Jelinek, W.R. and Molloy, G.R. (1973). Science, 181, 1215-1221.

Darnell, J.E., Philipson, L., Wall, R. and Adesnik, M. (1971). Science, 174, 507-510.

Denisova, L. Ya., Pustoshilova, N.M. and Zagrebelny, N. (1975). FEBS Lett., 56, 92-95.

Desai, L.S., Short, D.L., Friedman, O.M. and Foley, G.E. (1974). Eur. J. Biochem., 47, 453-460.

Deutscher, M.P. and Kornberg, A. (1969). J. Biol. Chem., 244, 3029-3037.

Diez, J. and Brawerman, G. (1974). Proc. Natl. Acad. Sci. U.S.A., 71, 4091-4095.

Dina, D., Meza, I. and Crippa, M. (1974). Nature, 248, 486-490.

Dobrzanska, M. and Buchowicz, J. (1976). Biochim. Biophys. Acta, 432, 73-79.

Downey, K.M., Byrnes, J.J., Jurmark, B.S. and So, A.G. (1973). Proc. Natl. Acad. Sci. U.S.A., 70, 3400-3404.

Dravid, A.R., Pete, N. and Mandel, P. (1971). J. Neurochem., 18, 299-306.

Edmonds, M. (1965). J. Biol. Chem., 240, 4621-4628.

Edmonds, M. and Abrams, R. (1960). Federation Proc., 19, 317.

Edmonds, M. and Abrams, R. (1962). J. Biol. Chem., 237, 2636-2642.

Edmonds, M. and Abrams, R. (1963). J. Biol. Chem., 238, 1186-1187.

Edmonds, M., Vaughan, M.H. and Nakazato, H. (1971). Proc. Natl. Acad. Sci. U.S.A., 68, 1336-1340.

Eoyang, L. and August, J.T. (1974). In "Comprehensive Virology" (Fraenkel-Conrat, H. and Wagner, R.R., Eds.), Vol. 2, pp 1-59, Plenum Press, London.

Feix, G. (1976). Nature, 259, 593-594.

Feix, G. and Hake, H. (1975). Biochem. Biophys. Res. Commun., 65, 503-509.

Fox, C.F., Robinson, W.S., Haselkorn, R. and Weiss, S.B. (1964). J. Biol. Chem., 239, 186-195.

Fraenkel-Conrat, H. (1956). J. Am. Chem. Soc., 78, 882-883.

Gander, E.S., Stewart, A.G., Morel, C.M. and Scherrer, K. (1973). Eur. J. Biochem., 38, 443-452.

Gierer, A. and Schramm, G. (1956). Nature, 177, 702-703.

Gillespie, D. and Gallo, R.C. (1975). Science, 188, 802-811.

Gorski, J., Morrison, M.R., Merkel, C.G. and Lingrel, J.B. (1974). J. Mol. Biol., 86, 363-371.

Gottesman, M.E., Canellakis, Z.N. and Canellakis, E.S. (1962). Biochim. Biophys. Acta, 61, 34-42.

Green, A.A. and Hughes, W.L. (1955). In "Methods in Enzymology" (Colowick, S.P. and Kaplan, N.O., Eds.), Vol. 1, pp. 67-138, Academic Press, New York.

Greenberg, J.R. and Perry, R.P. (1972). J. Mol. Biol., 72, 91-98.

Gulati, S.C., Kacian, D.L. and Spiegelman, S. (1974). Proc. Natl. Acad. Sci. U.S.A., 71, 1035-1039.

Gurgo, C., Rutigliano, B., Pagliuca, N. and Giuditta, A. (1970). Biochim. Biophys. Acta, 198, 31-44.

Hadidi, A. and Sethy, V.S. (1976). Biochim. Biophys. Acta, 425, 95-109.

Hall, R.H. (1963). Biochim. Biophys. Acta, 68, 278-283.

Harbers, E. and Heidelberger, C. (1959). Biochim. Biophys. Acta, 35, 381-388.

Hardy, S.J.S. and Kurland, C.G. (1966). Biochemistry, 5, 3668-3676.

Haruna, I., Ohno, T., Hozumi, N. and Watanabe, I. (1972). Gann Monograph on Cancer Research, 12, 203-211.

Hecht, L.I., Zamecnik, P.C., Stephenson, M.L. and Scott, J.F. (1958). J. Biol. Chem., 233, 954-963.

Henshaw, E.C. (1968). J. Mol. Biol., 36, 401-411.

Herbert, E. (1958). Federation Proc., 17, 241.

Herman, R.C., Williams, J.G. and Penman, S. (1976). Cell, 7, 429-437.

Hozumi, N., Haruna, I., Watanabe, I., Mikoshiba, K. and Tsukada, Y. (1975). Nature, 256, 337-339.

Huez, G., Marbaix, G., Hubert, E., Leclercq, M., Nudel, U., Soreq, H., Salomon, R., Lebleu, B., Revel, M. and Littauer, U.Z. (1974). Proc. Natl. Acad. Sci. U.S.A., 71, 3143-3146.

Hyatt, E.A. (1967). Biochim. Biophys. Acta, 142, 254-262.

Itoh, T., Haruna, I. and Watanabe, I. (1975). Nature, 257, 327-329.

Iwakura, Y. (1976). J. Biochem., 79, 61-68.

Jacob, S.T. (1973). Prog. Nuc. Acid Res. Mol. Biol., 13, 93-126.

Jacob, S.T., Sajdel, E.M., Muecke, W. and Munro, H.N. (1970). Cold Spring Harbor Symp. Quant. Biol., 35, 681-691.

Jacob, S.T. and Schindler, D.G. (1972). Biochem. Biophys. Res. Commun., 48, 126-134.

Jelinek, W., Adesnik, M., Salditt, M., Sheiness, D., Wall, R., Molloy, G., Philipson, L. and Darnell, J.E. (1973). J. Mol. Biol., 75, 515-532.

Kanehisa, T., Oki, Y. and Ikuta, K. (1974). Arch. Biochem. Biophys., 165, 146-152.

Kedinger, C., Gniazdowski, M., Mandel, Jr., J.L., Gissinger, F. and Chambon, P. (1970). Biochem. Biophys. Res. Commun., 38, 165-171.

Kish, V.M. and Pederson, T. (1975). J. Mol. Biol., 95, 227-238.

Kish, V.M. and Pederson, T. (1976). J. Biol. Chem., 251, 5888-5894.

Kish, V.M. and Pederson, T. (1977). Proc. Natl. Acad. Sci. U.S.A., 74, 1426-1430.

Klemperer, H.G. (1963a). Biochim. Biophys. Acta, 72, 416-420.

Klemperer, H.G. (1963b). Biochim. Biophys. Acta, 72, 403-415.

Kelmperer, H.G. (1964). Biochem. Biophys. Res. Commun., 15, 269-272.

Klemperer, H.G. (1965). Biochim. Biophys. Acta, 95, 251-261.

Klenow, H. and Overgaard-Hansen, K. (1970). FEBS Lett., 6, 25-27.

Klenow, H., Overgaard-Hansen, K. and Patkar, S.A. (1971). Eur. J. Biochem., 22, 371-381.

Klett, R.P., Cerami, A. and Reich, E. (1968). Proc. Natl. Acad. Sci. U.S.A., 60, 943-950.

Krakow, J.S. and Ochoa, S. (1963). Proc. Natl. Acad. Sci. U.S.A., 49, 88-94.

Küppers, B. and Sumper, M. (1975). Proc. Natl. Acad. Sci. U.S.A., 72, 2640-2643.

Lee-Huang, S., Sierra, J.M., Filipowicz, W. and Ochoa, S. (1976). Federation Proc., 35, 1728.

Lehninger, A. (1975). In "Biochemistry", 2nd Ed., North Publishers Inc., New York.

Levintow, L. (1974). In "Comprehensive Virology" (Fraenkel-Conrat, H. and Wagner, R.R., Eds.), Vol. 2, pp. 109-169, Plenum Press, London.

Levy, C.C., Schmukler, M., Frank, J.J., Karpetsky, T.P., Jewett, P.B., Hieter, P.A., LeGendre, S.M. and Dorr, R.G. (1975). Nature, 256, 340-342.

Lewin, B. (1974). In "Gene Expression", Vol. 2, pp. 288-298, John Wiley and Sons, London.

Lindell, T.J., Weinberg, F., Morris, P.W., Roeder, R.G. and Rutter, W.J. (1970). Science, 170, 447-449.

Lodish, H.F., Jacobson, A., Firtel, R., Alton, F. and Tuchman, J. (1974). Proc. Natl. Acad. Sci. U.S.A., 71, 5103-5108.

Longacre, S.S. and Rutter, W.J. (1977). J. Biol. Chem., 252, 273-283.

Louis, B.G. and Fitt, P.S. (1972). Biochem. J., 128, 755-762.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem., 193, 265-275.

Mandel, P., Dravid, A.R. and Pete, N. (1967). J. Neurochem., 14, 301-306.

McCaffrey, R., Smoler, D.F. and Baltimore, D. (1973). Proc. Natl. Acad. Sci. U.S.A., 70, 521-525.

Mikoshiba, K., Tsukada, Y., Haruna, I. and Watanabe, I. (1974). Nature, 249, 445-448.

Milanino, R. and Chargaff, E. (1973). Proc. Natl. Acad. Sci. U.S.A., 70, 2558-2562.

Mizuochi, T. and Fukasawa, H. (1976). J. Biochem., 79, 53-60.

Molloy, G.R., Jelinek, W., Salditt, M. and Darnell, J.E. (1974). Cell, 1, 43-53.

Molloy, G.R., Thomas, W.L. and Darnell, J.E. (1972). Proc. Natl. Acad. Sci. U.S.A., 69, 3684-3688.

Molnar, J., Besson, J. and Samarina, O.P. (1975). Molecular Biology Reports, 2, 11-17.

Monckton, R.P. and Naora, H. (1974). Biochim. Biophys. Acta, 335, 139-154.

Moulé, Y. and Chauveau, J. (1968). J. Mol. Biol., 33, 465-481.

Muthukrishnan, S., Filipowicz, W., Sierra, J.M., Both, G.W., Shatkin, A.J. and Ochoa, S. (1975). J. Biol. Chem., 250, 9336-9341.

Nakazato, H., Venkatesan, S. and Edmonds, M. (1975). Nature, 256, 144-146.

Naora, H. (1975). Cell Structure and Function, 1, 61-70.

Neuhoff, V., Schill, W.-B. and Jacherts, D. (1970). Hoppe-Seyler's Z. Physiol. Chem., 351, 157-162.

Niessing, J. (1975). Eur. J. Biochem., 59, 127-135.

Niessing, J. and Sekeris, C.E. (1971). FEBS Lett., 18, 39-42.

Niessing, J. and Sekeris, C.E. (1972). FEBS Lett., 22, 83-88.

Niessing, J. and Sekeris, C.E. (1973). Nature New Biology, 243, 9-12.

Niessing, J. and Sekeris, C.E. (1974). Biochem. Biophys. Res. Commun., 60, 673-680.

Niles, E.G., Conlon, S.W. and Summers, W.C. (1974). Biochemistry, 13, 3904-3912.

Niyogi, S.K. and Stevens, A. (1965a). J. Biol. Chem., 240, 2587-2592.

Niyogi, S.K. and Stevens, A. (1965b). J. Biol. Chem., 240, 2593-2598.

Obinata, M., Nasser, D.S. and McCarthy, B.J. (1975). Biochem. Biophys. Res. Commun., 64, 640-647.

Ohasa, S. and Tsugita, A. (1972). Nature New Biology, 240, 35-38.

Ohasa, S. and Tsugita, A. (1976). J. Mol. Biol., 105, 545-565.

Ohasa, S., Tsugita, A. and Mii, S. (1972). Nature New Biology, 240, 39-41.

Pace, N.R., Haruna, I. and Spiegelman, S. (1968). In "Methods in Enzymology" (Colowick, S.P. and Kaplan, N.O., Eds.), Vol. 12 (B), pp. 540-555, Academic Press, New York.

Page, M.G., Haynes, G.R. and Klemperer, H.G. (1967). Biochem. J., 102, 181-188.

Palmenberg, A. and Kaesberg, P. (1974). Proc. Natl. Acad. Sci. U.S.A., 71, 1371-1375.

Payne, K.J. and Boezi, J.A. (1970). J. Biol. Chem., 245, 1378-1387.

Priess, J., Dieckmann, M. and Berg, P. (1961). J. Biol. Chem., 236, 1748-1757.

Reid, B.D. and Parsons, P. (1971). Proc. Natl. Acad. Sci. U.S.A., 68, 2830-2834.

Ro-Choi, T.S. and Busch, H. (1974). In "The Cell Nucleus" (Busch, H., Ed.), Vol. 3, pp. 151-208, Academic Press, London.

Roeder, R.G. (1976). In "RNA Polymerase" (Losick, R. and Chamberlin, M., Eds.), pp. 312-314, Cold Spring Harbor Laboratory, U.S.A.

Roeder, R.G. and Rutter, W.J. (1969). Nature, 224, 234-237.

Rose, K.M. and Jacob, S.T. (1974). Biochem. Biophys. Res. Commun., 60, 390-397.

Saito, K. and Mitsuhashi, S. (1973). Jap. J. Microbiol., 17, 117-121.

Samarina, O.P., Lukanidin, E.M., Molnar, J. and Georgiev, G.P. (1968). J. Mol. Biol., 33, 251-263.

Sasaki, R., Goto, H., Arima, K. and Sasaki, Y. (1974a). Biochim. Biophys. Acta, 366, 435-442.

Sasaki, Y., Goto, H., Wake, T. and Sasaki, R. (1974b). Biochim. Biophys. Acta, 366, 443-453.

Schäfer, R., Zillig, W. and Priess, H. (1972). FEBS Lett., 25, 87-90.

Schreir, M.H. and Staehelin, T. (1973). J. Mol. Biol., 73, 329-349.

Seifart, K.H. and Benecke, B.J. (1975). Eur. J. Biochem., 53, 293-300.

Seifart, K.H., Benecke, B.J. and Juhasz, P.P. (1972). Arch. Biochem. Biophys., 151, 519-532.

Setlow, P. and Kornberg, A. (1972). J. Biol. Chem., 247, 232-240.

Siegel, A. and Hariharasubramanian, V. (1974). In "Comprehensive Virology" (Fraenkel-Conrat, H. and Wagner, R.R., Eds.), Vol. 2, pp. 61-108, Plenum Press, London.

Smith, R.G. and Gallo, R.C. (1972). Proc. Natl. Acad. Sci. U.S.A., 69, 2879-2884.

Smuckler, E.A. and Tata, J.R. (1972). Biochem. Biophys. Res. Commun., 49, 16-22.

Sobell, H.M. (1974). Sci. Amer., 231 (2), 82-91.

Spirin, A.S. (1969). Eur. J. Biochem., 10, 20-35.

Stein, H. and Hausen, P. (1970). Cold Spring Harbor Symp. Quant. Biol., 35, 709-717.

Temin, H.M. and Mizutani, S. (1970). Nature, 226, 1211-1213.

Tsiapalis, C.M., Dorson, J.W., DeSante, D.M. and Bollum, F.J. (1973). Biochem. Biophys. Res. Commun., 50, 737-743.

Twu, J.S. and Bretthauer, R.K. (1971). Biochemistry, 10, 1576-1582.

Verma, I.M. and Baltimore, D. (1974). In "Methods in Enzymology" (Grossman, L. and Moldave, K., Eds.), Vol. 29, pp. 125-130, Academic Press, New York.

Wehrli, W. and Staehelin, M. (1971). Bacteriol. Rev., 35, 290-309.

Wilkie, N.M. and Smellie, R.M.S. (1968a). Biochem. J., 109, 229-238.

Wilkie, N.M. and Smellie, R.M.S. (1968b). Biochem. J., 109, 485-494.

Williamson, R., Crossley, J. and Humphries, S. (1974). Biochemistry, 13, 703-707.

Winters, M.A. and Edmonds, M. (1973). J. Biol. Chem., 248, 4763-4768.

Wykes, J.R. and Smellie, R.M.S. (1966). Biochem. J., 99, 347-355.

Yu, F.L. (1976). Life Sciences, 18, 1171-1176.

Zamecnik, P.C., Stephenson, M.L., Scott, J.F. and Hoagland, M.B. (1957). Federation Proc., 16, 275.